(19) DANMARK

(10) DK/EP 2502983 T3

(12) Oversættelse af europæisk patentskrift

Patent- og Varemærkestyrelsen

(51) Int.Cl.: *C 12 N 1/12 (2006.01)* *A 01 N 63/00 (2006.01)* *A 61 K 31/137 (2006.01)*
A 61 K 31/27 (2006.01) *A 61 K 31/415 (2006.01)* *A 61 K 31/4164 (2006.01)*
A 61 K 31/4174 (2006.01) *A 61 K 31/496 (2006.01)* *A 61 K 35/748 (2015.01)*
C 12 N 1/00 (2006.01) *C 12 N 1/06 (2006.01)* *C 12 N 9/42 (2006.01)*
C 12 N 9/80 (2006.01)

(45) Oversættelsen bekendtgjort den: **2017-09-25**

(80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2017-06-07**

(86) Europæisk ansøgning nr.: **12157835.5**

(86) Europæisk indleveringsdag: **2005-11-03**

(87) Den europæiske ansøgnings publiceringsdag: **2012-09-26**

(30) Prioritet: **2004-11-03 AU 2004906317**

(62) Stamansøgningsnr: **05799363.6**

(84) Designerede stater: **AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IS IT LI LT LU LV MC NL PL PT RO SE SI SK TR**

(73) Patenthaver: **Biovite Australia Pty Ltd, 1/45 - 47 Township Drive, West Burleigh, Queensland 4219, Australien**

(72) Opfinder: **DUNCAN, Kelvin Winston, 28B Lodge Place Avonhead, Christchurch, New Zealand**
JOHNSTON, Peter Owen, 44 Meadow Peak, 85 Palm Meadows Drive, Carrara, Queensland 4211, Australien
Brown, Ashley Michael, c/o Biovite Australia Pty Ltd., 1/45-47 Township Drive, West Burleigh, Queensland 4219, Australien

(74) Fuldmægtig i Danmark: **RWS Group, Europa House, Chiltern Park, Chiltern Hill, Chalfont St Peter, Bucks SL9 9FG, Storbritannien**

(54) Benævnelse: **ARTHROSPIRA-BASEREDE SAMMENSÆTNINGER OG ANVENDELSER DERAF**

(56) Fremdragne publikationer:
EP-A- 0 629 397
WO-A-98/54987
WO-A1-98/25470
WO-A1-98/25471
JP-A- 9 067 265
WIKIPEDIA: "Spirulina", WIKIPEDIA , 11 August 2009 (2009-08-11), XP002541990, Internet Retrieved from the Internet: URL:http://en.wikipedia.org/wiki/Spirulina _(dietary_supplement) [retrieved on 2009-08-18]
HIDENORI SHIMAMATSU: "Mass production of Spirulina, an edible microalga", HYDROBIOLOGIA, KLUWER ACADEMIC PUBLISHERS, DO, vol. 512, no. 1-3, 1 January 2004 (2004-01-01), pages 39-44, XP019244647, ISSN: 1573-5117
KONTOYIANNIS D P ET AL: "Combination chemotherapy for invasive fungal infections: What laboratory and clinical studies tell us so far", DRUG RESISTANCE UPDATES 200310 GB, vol. 6, no. 5, October 2003 (2003-10), pages 257-269, XP002541991, ISSN: 1368-7646

Fortsættes ...

KONTOYIANNIS D P ET AL: "Toward more effective antifungal therapy: The prospects of combination therapy", BRITISH JOURNAL OF HAEMATOLOGY 200407 GB, vol. 126, no. 2, July 2004 (2004-07), pages 165-175, XP009121633, ISSN: 0007-1048
JOHN D. BARANOWSKI ET AL: "Effects of drying on selected qualities of Spirulina platensis protein", JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY, vol. 32, no. 6, 1 November 1984 (1984-11-01), pages 1385-1387, XP55035140, ISSN: 0021-8561, DOI: 10.1021/jf00126a042
DESMORIEUX H. AND HERNANDEZ F.: "Biochemical and Physical criteria of Spirulina after different drying processes", DRYING 2004 - PROCEEDINGS OF THE 14TH INTERNATIONAL DRYING SYMPOSIUM (IDS 2004), vol. B, 2004, pages 900-907, XP002681813,
J. F. MANEN: "The cpcB--cpcA locus as a tool for the genetic characterization of the genus Arthrospira (Cyanobacteria): evidence for horizontal transfer", INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY, vol. 52, no. 3, 1 May 2002 (2002-05-01), pages 861-867, XP55035378, ISSN: 1466-5026, DOI: 10.1099/ijs.0.01981-0
Tomaselli, Luisa: "Morphology, Ultrastructure and Taxonomy of Arthrospira (Spirulina) maxima and Arthrospira (Spirulina) platensis"; "Chapter 1" In: Avigad Vonshak: "Spirulina platensis (Arthrospira): Physiology, Cell-biology and Biotechnology", 1997, Taylor & Francis, Great Britain, XP002681814, pages 1-15, * the whole document *
TADROS M G ET AL: "Yield and quality of cyanobacteria Spirulina maxima in continuous culture in response to sodium chloride", APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY, HUMANA PRESS, INC, UNITED STATES, vol. 51-52, 1 January 1995 (1995-01-01), pages 275-281, XP009121554, ISSN: 0273-2289, DOI: 10.1007/BF02933430
ARMIDA HERNÁNDEZ-CORONA ET AL: "Antiviral activity of Spirulina maxima against herpes simplex virus type 2", ANTIVIRAL RESEARCH, ELSEVIER BV, NL, vol. 56, no. 3, 1 December 2002 (2002-12-01), pages 279-285, XP009121553, ISSN: 0166-3542, DOI: 10.1016/S0166-3542(02)00132-8 [retrieved on 2002-10-02]

DESCRIPTION

[0001] The present invention relates to a topical biocidal composition comprising a biocidally effective amount of physiologically stressed *Arthrospira,* a method for preparing such a composition for topical use on a subject and a biocidally effective amount of physiologically stressed *Arthrospira* for topical administration to a subject for use in preventing or treating a bacterial infection or infestation of the subject, or in repairing or preventing a skin defect of a mammal.

[0002] *Arthrospira* (formerly known as *Spirulina*) is a cyanobacteria that is grown primarily for use as a food and/or protein source. Therapeutic uses for *Arthrospira* have also been described, including use as an anti-viral agent, anti-cancer agent, reducer of cholesterol, reducer of diabetes, reducer of hypertension and immunomodulator. See, for example, the references cited at the Spirulina Source.com website at http://www.spirulinasource.com/library.html.

[0003] The document WO 1998/025471 discloses a method of preparation of a treated Spirulina compound, which method includes the steps of rehydrating desiccated Spirulina, stressing the culture and freeze drying the composition to a powder. The resultant compound has activity as an anti-viral compound and an anti-bacterial compound.

[0004] An object of the present invention is to provide an *Arthrospira* composition for use as a biocide and/or therapeutic.

Physiologically Stressed *Arthrospira* has Biocidal Activity

[0005] The present inventors have discovered that *Arthrospira,* when physiologically stressed, produces at least one type of bioactive agent that is active against fungi, bacteria and presumably viruses. *Arthrospira* can be physiologically stressed, for example, by depriving the organism of nutrients or light, or by dehydrating/drying the organism. A sign of an *Arthrospira* filament (trachoma) being "physiologically stressed" is when it enters an anabiotic state, in which state the filament rounds up and a thick mucoid coating is produced. The filament and/or thick mucoid coating is believed by the inventors to contain one or more bioactive agents that protect the organism against predators. One or more of the bioactives may break down the cell wall or exoskeleton of a predatory organism. One or more of the bioactive agents may be, for example, a lytic or modifying agent such as a chitinase, chitosanase or chitin deacetylase, that digests chitin, a chitin derivative (such as chitosan) or other cell wall polymer having, for example, N-acetylglucosamine or D-glucosamine as a polymer subunit. Peptidoglycan of Gram-positive bacteria is one such polymer, comprising N-acetylglucosamine and N-acetylmuramic acid.

[0006] The chitin, chitin derivative or polymer can be in any suitable form. The chitin, chitin

derivative or polymer can be in a substantially purified form or can be part of an organism such the cell wall of a fungus or Gram-positive bacteria, or part of the exoskeleton of an insect.

[0007] Preferably, the bioactive agent may be a chitinase, chitosanase or chitin deacetylase and the test substrate is chitin, a chitin derivative or a polymer having N-acetyl-D-glucosamine as a polymer subunit. Preferably, respirometry is used to assay modification (ie. degradation) of the chitin, chitin derivative or other polymer.

[0008] The present invention relates to a method for preparing a biocidal composition for topical use on a subject, said method comprising the steps of: (1) physiologically stressing *Arthrospira* by lack of nutrients or light, by dehydrating, or by removing up to 80 % of liquid within which the *Arthrospira* is being grown; and (2) combining a biocidally effective amount of the stressed *Arthrospira* of (1) with a carrier, solvent, base or excipient suitable for topical administration to the subject, wherein the biocidally effective amount is effective against bacteria of the subject, or skin defects of a mammal; and wherein the *Arthrospira* has not been freeze dried.

[0009] Preferred embodiments of said method are claimed in dependent claims 2 to 4 and 14.

[0010] The present invention also relates to a topical biocidal composition comprising a biocidally effective amount of physiologically stressed *Arthrospira,* wherein the physiological stressing is achieved by lack of nutrients or light, by dehydrating, or by removing up to 80 % of liquid within which the *Arthrospira* is being grown, and a carrier, solvent, base or excipient suitable for topical administration to a subject, wherein the biocidally effective amount is effective against bacteria of the subject, or skin defects of a mammal; and wherein the *Arthrospira* is not freeze dried.

[0011] Preferred embodiments of said composition are claimed in claims 6, 7 and 13.

[0012] The invention finally relates to a biocidally effective amount of physiologically stressed *Arthrospira,* wherein the physiological stressing is achieved by lack of nutrients or light, by dehydrating, or by removing up to 80 % of liquid within which the *Arthrospira* is being grown, and a carrier, solvent, base or excipient for topical administration to a subject for use in preventing or treating a bacterial infection or infestation of the subject, or in repairing or preventing a skin defect of a mammal; wherein the *Arthrospira* is not freeze dried.

[0013] Preferred embodiments of said biocidally effective amount of physiologically stressed *Arthrospira* are claimed in claims 9 to 12.

Use of Physiologically Stressed *Arthrospira* as a Biocide and/or Therapeutic

[0014] Chitin, chitin derivatives and glucosamine containing polymers are found throughout nature, usually as a component of a cell wall or exoskeleton of, for instance: algae, fungi,

protozoans, ciliates, coelenterates, bryozoans, molluscs, annelids, arthropods, branchiopods, phoronida, pogonophora, and bacteria (Gram-positive bacteria and *Rhizobia*).

[0015] Hence, physiologically stressed *Arthrospira* having lytic or modifying activity against chitin, chitin derivatives and glucosamine containing polymers is potentially capable of being used as a biocide against a broad range of organisms. "Biocide" as used herein refers to either the killing of an organism or inhibition of the growth or replication of an organism (ie. the organism may not actually be killed). It is to be appreciated that some applications of the biocide will be for therapeutic purposes.

[0016] According to the present invention, there is provided a topical biocidal composition comprising a biocidally effective amount of physiologically stressed *Arthrospira,* wherein the physiological stressing is achieved by lack of nutrients or light, by dehydrating, or by removing up to 80 % of liquid within which the *Arthrospira* is being grown, and a carrier, solvent, base or excipient suitable for topical administration to a subject, wherein the biocidally effective amount is effective against bacteria of the subject, or skin defects of a mammal; and wherein the *Arthrospira* is not freeze dried

[0017] According to the present invention, there is provided a method for preparing a biocidal composition for topical use on a subject, said method comprising the steps of: (1) physiologically stressing *Arthrospira* by lack of nutrients or light, by dehydrating, or by removing up to 80 % of liquid within which the *Arthrospira* is being grown; and (2) combining a biocidally effective amount of the stressed *Arthrospira* of (1) with a carrier, solvent, base or excipient suitable for topical administration to the subject, wherein the biocidally effective amount is effective against bacteria of the subject, or skin defects of a mammal; and wherein the *Arthrospira* has not been freeze dried.

[0018] Preferably, the step of physiologically stressing *Arthrospira* comprises removing up to 80 % of liquid within which the *Arthrospira* is being grown; and step (2) is preceded by steps (1a) and (1b) as follows: (1a) washing the stressed *Arthrospira* in order to remove contaminants; and (1b) drying the washed *Arthrospira.*

[0019] Preferably, the subject is a human, an animal, an agricultural or horticultural product, soil, a man-made structure, or a waterway.

[0020] Preferably, the skin defect is selected from the group consisting of acne damage, rosacea, a reddened area, a crack, a burn, a blister, psoriasis, eczema, scaling, wrinkles, a stomatitis, a lesion, a pustule, a wound, cradle cap, diaper rash, an ulcer, a cold sore, shaving rash, dermatitis, and cracked heels and elbows.

[0021] Surprisingly, the inventors have found that taking *Arthrospira* from its growth environment may be enough for physiological stressing to occur and for inducing expression of one or more bioactive agents having biocidal activity. That is, steps for physiologically stressing *Arthrospira* as taught in the specifications of New Zealand Patents No. 336620 and 336619

need not be carried out and the *Arthrospira* need not be further processed (disrupted, explosively decompressed, potentiated, milled, rehydrated in sodium bicarbonate or freeze dried) in order for there to be biocidal activity.

[0022] Furthermore, all commercially available preparations (tablets, capsules and powders) of *Arthrospira* that have thus far been tested by the inventors have been found to have activity against a substrate, and that activity is believed to be indicative of biocidal activity. Such preparations are usually prepared by harvesting the *Arthrospira,* washing and drying the *Arthrospira,* without a step for specifically stressing the organism. In fact, commercial growers of *Arthrospira* tend to minimise stressing of the organism as much as possible as the thick mucoid coating tends to clog machinery for processing the organism.

[0023] For any one or more of the compositions described herein, the *Arthrospira* can be any suitable species, or mixture of species or *Arthrospira* variants including, but not limited to, *A. maxima.* It is to be pointed out that *Arthrospira* and *Spirulina* are two separate genera. The separation between these two genera has been repeatedly affirmed on the basis of many characteristics including: helicity and trichome size, cell wall structure and pore pattern, gas vacuoles, thylakoid pattern, trichome motility and fragmentation, GC content, oligonucleotide catalogue of 16S rRNA, and mutations in the cpcB--cpcA locus. See, for instance, J. F. Manen and J. Falquet (2002) "The cpcB--cpcA locus as a tool for the genetic characterization of the genus Arthrospira (Cyanobacteria): evidence for horizontal transfer". International Journal of Systematic and Evolutionary Microbiology, Vol 52, 861-867; and, Chapter 1: Morphology, Ultrastructure and Taxonomy of Arthrospira (Spirulina) Maxima and Arthrospira (Spirulina) Platensis by Luisa Tomaselli, of Spirulina platensis (Arthrospira): Physiology, Cell-biology and Biotechnology. Taylor and Francis. Avigad Vonshak (Ed) 1997. The compositions described in this specification exclude species of the *Spirulina* genus.

[0024] The composition can comprise intact *Arthrospira* filaments, segments of the filaments, disrupted/lysed segments or extracts/fractions thereof. The composition can comprise *Arthrospira* that is alive or that is not alive. Preferably, the composition comprises disrupted *Arthrospira* filaments and segments.

[0025] The *Arthrospira*-based composition can be prepared in any suitable way, provided that the biocidal activity is not compromised. Normally, this would involve the steps of: (I) growing *Arthrospira* in any suitable way; (II) harvesting the grown *Arthrospira* in any suitable way; and (III) drying the *Arthrospira* in any suitable way. Optional steps include: additionally physiologically stressing the *Arthrospira* in any suitable way; disrupting the filaments and segments in any suitable way, sterilising the organism in any suitable way, decolourising the *Arthrospira* in any suitable way, removing or degrading the chlorophyll in any suitable way, and milling the dried *Arthrospira.*

[0026] Some of the above steps are described, for example, in the specifications of New Zealand Patents No. 336620 and 336619. For example, stressing can be carried out at the time of harvest by either depriving the *Arthrospira* of essential nutrients (nutrient diminution) or

light for a period sufficient to stress it but not kill it by management of the growing conditions. Stressing can also be by partial desiccation or by harvesting the *Arthrospira* and keeping it alive in damp conditions until stressed but not dead. For example, *Arthrospira* can be spray dried to form a fine powder of disrupted cells. Spray drying may take place, for example, at 50-190°C for a few seconds. For example, drying can be carried out in any suitable way, including by cyclonic drying, heat pump drying, heat tube drying, refractory drying or thermal drying under about 67°C for short periods of time. For example, filaments can be disrupted using milling or explosive decompression as described in New Zealand Patents No. 328013 and No. 328740.

[0027] The concentration of *Arthrospira* in the composition can vary depending on the application, eg. anywhere from about 0.01% to about 100%. The composition can include any suitable solvent, carrier, base, excipient, filler, binder, plasticiser, emulsifier, stabiliser, lubricant, buffering agent, emollient, solubilising agent, suspending agent, thickener, fragrance, colourant and preservative.

[0028] Depending on the intended use, the composition can further comprise one or more actives not derived from *Arthrospira,* such as antimicrobials (e.g. bactericides or antifungals), therapeutic agents (e.g. wound healers, steroids) or general nutrients (e.g. amino acids, vitamins). Such actives are well known in the art.

[0029] The composition can be in any suitable form. The composition can be administered or applied in any suitable way. The composition can be a liquid, gel or solid, or a mixture thereof. The composition can be a liquid culture of *Arthrospira* which is applied as a spray. Alternatively, the composition can be a powder which is evenly distributed over a target site by shake or puffer application. The infected or infested subject can be dipped into a bath containing a liquid form of the composition. The composition and method of application or administration can be as described in the specifications of New Zealand Patents No. 336620 and 336619.

[0030] For therapeutic use, the composition can be administered or applied, for example, orally, parenterally, by inhalation spray, topically, rectally, nasally, buccally or vaginally. The composition can be a tablet, solution, aerosol, spray, cream, ointment, lotion, emulsion, gel or powder. The composition can be in the form of an *Arthrospira*-impregnated bandage, dressing, adhesive plaster, suppository, pessary or poultice.

[0031] Preferably, the biocide is active against fungi, Gram-positive bacteria, protozoa, viruses, crustaceans, acari and insects, some of which are addressed in more detail below.

Use of Physiologically Stressed *Arthrospira* as an Antifungal

[0032] Physiologically stressed *Arthrospira* may be used to treat at least the following types of fungi: *Zygomycotina; Ascomycotina; Fungi Imperfecti; Malassezia; Microsporum; Trichophyton;* and *Epidermophyton.*

[0033] Surprisingly, the present inventors have found that physiologically stressed *Arthrospira* interacts synergistically with monographed anti-fungal actives (fungi-static agents) to provide superior broad spectrum antifungal activity. The inventors have found that stressed *Arthrospira* can inhibit the growth of fungi, kill fungi and prevent regrowth of fungi.

Use of Physiologically Stressed *Arthrospira* as an Antibacterial

[0034] Physiologically stressed *Arthrospira* has been found by the inventors to be an effective antibacterial against Gram-positive bacteria. The mode of antibacterial action is not clearly understood. It is possible that cell walls of Gram-positive bacteria are lysed or otherwise modified by one or more bioactives from *Arthrospira.* The cell walls contain peptidoglycan (comprising N-acetylglucosamine and N-acetylinuramic acid) and peptidoglycan may be digestible by one or more of the bioactives.

[0035] According to the invention, there is also provided the biocidally effective amount of physiologically stressed *Arthrospira* as described above, for use in preventing or treating a bacterial infection or infestation of the subject, or in repairing or preventing a skin defect of a mammal; wherein the *Arthrospira* is not freeze dried.

[0036] Preferably the subject is human and the bacterial infection is caused by *Propionibacterium acnes.*

Use of Physiologically Stressed *Arthrospira* as a Pesticide

[0037] The pesticide can be directed to pests such as crustacean and acari. The pesticide can be directed to insects such as flies (eg. *Simulidae* - black flies), wasps, mosquitoes and termites. The pesticide can be used to stop the transmission of parasites, such as those causing malaria, and other insects-borne diseases.

Use of *Arthrospira* as a Therapeutic

[0038] When using physiologically stressed *Arthrospira* as a biocide and/or therapeutic on skin, the inventors have found that one or more other substituents of *Arthrospira* interact synergistically with the one or more bioactive agents to promote healing of the skin. These other substituents are likely to include: beta-carotene, which provides nourishment to the skin; phycocyanin, which is an anti-inflammatory; other proteins; and other nutrients, including vitamins, minerals, trace elements, anti-oxidants, essential oils and carbohydrates.

[0039] First, the stressed *Arthrospira* inhibits or kills the microbe - be it a fungus, bacteria or

virus - and then other substituents of *Arthrospira* repair the damage caused by the microbe.

[0040] The present inventors have found that *Arthrospira* has therapeutic qualities even when not physiologically stressed, such as in the treatment of skin conditions and in skin repair.

[0041] The skin defect can be, for example, a pit, acne damage, rosacea, a reddened area, a crack, a burn, a blister, psoriasis, eczema, scaling, wrinkles, a papule, a stomatitis, a lesion, a pustule, a wound, cradle cap, diaper rash, an ulcer, a cold sore, shaving rash, chicken pox, dermatitis, cracked heels and elbows. The composition can be used to treat burns, insect and animal bites, and to remove inflammation of the skin. The composition can be used to reduce itchiness of the skin. The composition can be used to repair scar tissue, sun-damaged skin, and dry and scaly skin that has lost its elasticity.

[0042] Other aspects and embodiments of the invention will become apparent from the following detailed description thereof.

BRIEF DESCRIPTION OF THE FIGURES

[0043]

Figure 1 shows activity of physiologically stressed *Arthrospira* (AMYCOT® and unprocessed powder) on chitin as assessed by respirometry;

Figure 2 shows antifungal activity of AMYCOT® on *C. albicans;*

Figure 3 shows antibacterial activity of AMYCOT® on *P. acnes* as assessed by respirometry;

Figure 4 shows the antifungal activity of different commercial preparations of *Arthrospira* (Spirulina) on *T. rubrum;*

Figure 5 shows antifungal activity of a commercial preparation (Life Stream) of *Arthrospira* on *A. niger;*

Figure 6 shows antifungal activity of AMYCOT® on *M. canis*;

Figure 7 shows antifungal activity of AMYCOT® on *F. graminearum*;

Figure 8 shows antifungal activity of AMYCOT® on *M. furfur;*

Figure 9a shows antifungal activity of shampoo AMYCOT® on *T. rubrum;*

Figure 9b shows antifungal activity of shampoo AMYCOT® on *C. albicans;*

Figure 10 shows antifungal activity of AMYCOT® on *P. chartarum*;

Figure 11 shows antifungal activity of AMYCOT® on *B. cinerea;*

Figure 12 shows *T. rubrum* inhibitory results using AMYCOT® and various monographed anti-fungal actives;

Figure 13 shows *T. mentagrophytes* inhibitory results using AMYCOT® and various monographed actives;

Figure 14 shows *C. albicans* inhibitory results using AMYCOT® and various monographed actives;

Figure 15 shows *C. albicans* inhibitory results using a monographed active;

Figure 16 shows *T. mentagrophytes* inhibitory results using AMYCOT® in combination with a monographed active and various monographed actives;

Figure 17 shows *C. albicans* inhibitory results using AMYCOT® in combination with a monographed active and various monographed actives;

Figure 18 shows *T. mentagrophytes* inhibitory results using AMYCOT® in combination with monographed actives;

Figure 19 shows *T. mentagrophytes* inhibitory results using AMYCOT® in combination with monographed actives;

Figure 20 shows *T. mentagrophytes* inhibitory results using AMYCOT® in combination with a monographed active and various monographed actives;

Figure 21 shows *C. albicans* inhibitory results using AMYCOT® in combination with a monographed active and various monographed actives;

Figure 22 shows *T. mentagrophytes* fungicidal results using AMYCOT® in combination with monographed actives;

Figure 23 shows *T. rubrum* fungicidal results using AMYCOT® in combination with monographed actives;

Figure 24 shows *T. mentagrophytes* fungicidal results using AMYCOT® in combination with monographed actives;

Figure 25 shows *T. mentagrophytes* fungicidal results using AMYCOT® in combination with monographed actives;

Figure 26 shows *T. mentagrophytes* fungicidal results using AMYCOT® in combination with monographed actives;

Figure 27 shows *C. albicans* fungicidal results using AMYCOT® in combination with a monographed active and various monographed actives;

Figure 28 shows *T. rubrum* fungicidal results using AMYCOT® and monographed actives;

Figure 29 shows activity of physiologically stressed *Arthrospira* (AMYCOT®) on chitin at

different temperatures as assessed by respirometry;

Figure 30 shows activity of physiologically stressed *Arthrospira* (AMYCOT®) on chitin at various pH conditions as assessed by respirometry;

Figure 31 shows antifungal activity of *Arthrospira* on *T. mentagrophytes;*

Figure 32 shows antifungal activity of *Arthrospira* on *T. rubrum;*

Figure 33 shows antifungal activity of *Arthrospira* on *M. fructicola;* and

Figure 34 shows antifungal activity of "potentiated AMYCOT®" and "original AMYCOT®" on *Alternaria sp.*

BEST MODE AND OTHER MODES OF CARRYING OUT THE INVENTION

[0044] By way of illustration only, preferred embodiments of the present invention are described in detail, with reference to the following series of examples.

Example 1 - Preparation of Physiologically Stressed *Arthrospira*

[0045] A culture of *Arthrospira maxima* (obtainable from Biovite Australia Pty Ltd) was stressed by nutrient diminution or partial desiccation. The *Arthrospira* was dewatered and dried to form an "unprocessed" powder. Dried *Arthrospira* was then explosively decompressed (potentiated) using the method described in New Zealand Patents No. 328013 and No. 328740. The *Arthrospira* was re-hydrated, decolourised, dried and milled to form a dry "premix powder". The premix powder was then dispersed in a suitable carrier (eg. commercially available aqueous British Pharmacopoeia cream or water). The premix powder is referred to as "AMYCOT® premix powder".

Example 2 - Characterisation of Activity of Stressed *Arthrospira*

[0046] The results of respirometry suggest that physiologically stressed *Arthrospira* is capable of modifying or lysing substrates comprising chitin, chitosan and/or N-acetyl-D-glucosamine.

Chitin Substrate

[0047] Standard amounts of AMYCOT® premix powder and the unprocessed powder were separately reacted with four-unit polymer BHD chitin (Sigma, catalogue number C7170) in a

Warburg Respirometer using standard techniques. Each composition was mixed together with chitin and the quantity of gas evolved was measured. The reaction was monitored for one-hour following equilibration for 30 minutes. The same composition, but pre-boiled in a water bath for five minutes, did not produce any gas which suggested that the activity could be enzymic and could be due to one or more types of denaturable proteins.

[0048] The results are plotted in Figure 1. The rate of activity (presumably enzymatic activity) of unprocessed powder was 694.9 ml/g cellular material/g substrate/hour. The rate of activity of AMYCOT® premix powder was 729.65 ml/g cellular material /g substrate/hour.

[0049] Respirometry was also used to identify the optimum temperature for the activity. AMYCOT® premix powder (0.02 g and 0.06 g) was incubated with chitin (0.02 g) at 20°C, 30°C, 33°C and 40°C. AMYCOT® premix powder in a cream based (0.05 g) was also incubated with chitin (0.02 g) at those temperatures. The results are plotted in Figure 29. The optimum temperature for the activity in each case was determined to be 33°C.

[0050] Respirometry was also used to identify the optimum pH for the activity. AMYCOT® premix powder (0.02 g) was suspended in buffers of varying pH (60-70% ethanol, 30-40% water, 0-0.05% phenolphthalein, 0-0.03% bromothymol blue, 0-0.02% methyl red, sodium salt, 0-0.01% sodium hydroxide and 0-0.01% methyl orange, sodium salt at pH 5, 7, 9 and 11) and was incubated with chitin (0.02 g) at 33°C. The results are plotted in Figure 30. The optimum pH for activity was determined to be 6.

[0051] These results suggest that the bioactive agent or agents should have activity when topically applied to skin as skin has a similar temperature and pH.

N-acetyl-D-glucosamine and Chitosan Substrates

[0052] AMYCOT® premix powder (0.02g) was separately incubated with N-acetyl-D-glucosamine (0.02g, Sigma catalogue number A8625-5G) and chitosan (0.02g, Sigma catalogue number C3646) substrates in a Warburg Respirometer at 33°C. Each reaction was monitored for one-hour following equilibration for 30 minutes.

[0053] The results for AMYCOT® plus N-acetyl-D-glucosamine are presented in the table below.

Table 1 - AMYCOT® + N-acetyl-D-glucosamine

Time (min)	mm of gas	mm of gas	mm of gas
0	0	0	0
5	9	6	7
10	12	9	11
30	12	10	11

Table 1 - AMYCOT® + N-acetyl-D-glucosamine			
Time (min)	**mm of gas**	**mm of gas**	**mm of gas**
60	12	10	11

[0054] The results for AMYCOT® plus chitosan are presented in the table below.

Table 2 - AMYCOT® + Chitosan			
Time	**mm of gas**	**mm of gas**	**mm of gas**
0	0	0	0
5	12	10	10
10	14	12	14
30	17	13	15
60	17	14	16

[0055] For comparison, results for AMYCOT® (0.02g) plus chitin (0.02g), incubated at 33°C, are presented in the table below.

Table 3 - AMYCOT® + Chitin			
Time	**mm of gas**	**mm of gas**	**mm of gas**
0	0	0	0
5	12	10	15
10	18	18	20
30	19	18	21
60	19	19	21

[0056] These results suggest that physiologically stressed *Arthrospira* has one or more bioactive agents that lyse or modify chitin, chitosan and/or N-acetyl-D-glucosamine, or possibly another type of polymer having N-acetyl-D-glucosamine as a polymer subunit. The bioactive agent could be, for instance, a chitinase, chitosanase or chitin deacetylase.

Example 3 - Antifungal and Antibacterial Activity of Stressed *Arthrospira*

[0057] The biocidal activity of AMYCOT® premix powder was *tested in vitro* on live fungi and Gram-positive bacteria.

[0058] Live cultures of target pathogens were sourced from the American Type Culture Collection (ATCC), the Australian Collection of Microorganisms (ACM) and Sullivan Niccolaides.

The cultures that were tested are shown in Table 4.

Table 4

Pathogen	ATCC No.	ACM No.	Isolates
Trichophyton mentagrophytes	4808,9533	5068	
Candida albicans	753	4574	
Trichophyton rubrum			SN01 (Sullivan Niccolaides)
Epidermophyton floccosum			SN02 (Sullivan Niccolaides)
Propionibacterium acnes	25746	5109	

[0059] A uniform lawn of each pathogen was grown on a dish containing Potato Dextrose Agar (PDA) medium. After 3-4 days of incubation, four wells were cut in the PDA medium with a sterile 8 mm diameter cork borer. Measured amounts of AMYCOT® premix powder and control creams/powders were placed in each well. The dishes were cultured at conditions appropriate for the respective pathogen and the dishes were inspected daily.

[0060] After 2-4 days two concentric zones around the well were evident:

1. 1. A smaller transparent circular "clearance" zone around a well. This is an area where fungi have been destroyed, leaving only residual dry cytoplasmic matter (as seen by light and electron microscopy).
2. 2. A larger semitransparent "affected" zone extending from the perimeter of the clearance zone. The affected zone is recognised by the lesser height of the lawn, its lesser density and greater transparency compared with unaffected lawn. This is an area where fungi cell walls have been largely destroyed, but that some are still present, often as rounded cytoplasmic spheres (as seen by light and electron microscopy).

[0061] Measurement of the zones is taken from the outside perimeter of the well to the outside perimeter of the zone in each case. This measurement is referred to as the zonal distance (ZD). The radius of the zones is a non-linear measure of the efficacy of the biocidal agent/s against the pathogen *in vitro.*

[0062] The *in vitro* control results are shown in Table 5.

Table 5				
ACM No.	**Pathogen**	**Result**	**Size of clearance zone (ZD)** (mm)	**Size of affected zone** (mm)
5068	*Trichophyton mentagrophytes*	Clearance	6	10
4574		Clearance	6.5	-
5109	*Propionibacterium acnes* (cultured anaerobically)	No clearance	-	-
ATCC No.				
4808	*Trichophyton mentagrophytes*	Clearance	5	8
9533	*Trichophyton mentagrophytes*	Clearance	6	10
753	*Candida albicans*	Clearance	6.5	-
25746	*Propionibacterium acnes* (cultured anaerobically)	No clearance	-	-
Isolate No.				
SN01	*Trichophyton rubrum*	Clearance	6	-
SN02	*Epidermophyton floccosum*	Clearance	5	8
301	*Tinea pedis* - isolate	Clearance	6	10
307	Jockey itch-isolate	Clearance	6	10

[0063] Figure 2 shows clearance of *Canada albicans* (ACM 4574) using AMYCOT®, which is evidence of the efficacy of one or more biocidal agents against the condition thrush. AMYCOT® premix powder is at the 6 o'clock position and a "human trial cream" having 12.5% w/w AMYCOT® in an aqueous cream is at the 12 o'clock position (ZD of 9 mm).

[0064] In the case of the anaerobic acne-causing bacteria *Propionibacterium acnes,* no clearance was obtained when tested under anaerobic conditions. An explanation for this is provided in Example 4.

Example 4 - Antibacterial Activity of Stressed *Arthrospira* on *Propionibacterium acnes*

[0065] Respirometry showed that AMYCOT® has antibacterial activity against *P. acnes*.

[0066] AMYCOT® premix powder (0.02g) and 5g "human trial cream" comprising 12.5% w/w

AMYCOT® were separately reacted with a broth of the anaerobic acne-causing organism *P. acnes* (ACM No. 5109) in a Warburg Respirometer in the presence or absence of oxygen. The results were compared with those of a control comprising *P. acnes* broth devoid of AMYCOT®. In the absence of oxygen, no gas was liberated. In the presence of oxygen, gas was liberated and each composition had antibacterial activity.

[0067] The results for AMYCOT® and human trial cream in the presence of oxygen are plotted in Figure 3. The (presumably enzymatic) rate of AMYCOT® premix powder was 626.00 ml/g cellular material/g substrate/hour. The rate of human trial cream was 12.00 ml/g cellular material/g substrate/hour.

[0068] The results showed that in order for the composition to work as an antibacterial on *P. acnes in vivo,* the presence of oxygen is required. Therefore, at least one of the biocidal agents may be an oxidase.

[0069] The inventors suspect that one or more of the biocidal agents against fungi may lyse or modify the cellular wall of fungi. Such an agent may be a chitinase, chitosanase or chitin deacetylase.

[0070] Although *P. acnes* and other Gram-positive bacteria are not believed to have chitin or chitosan in their cellular walls, it is suspected that one or more biocidal agents lyse or modify N-acetyl-D-glucosamine/peptidoglycans in the bacterial cell wall.

Example 5 - Different Commercial Sources of *Arthrospira* have Antifungal Activity

[0071] Commercial preparations of dried *Arthrospira* (powders, tablets and capsules) were sourced from different suppliers in different jurisdictions and tested for antifungal activity. The powders are typically prepared by harvesting *Arthrospira,* washing the harvested *Arthrospira,* and drying the washed *Arthrospira*. Some suppliers mill the *Arthrospira* whilst wet. Preparation does not entail the step of specifically stressing *Arthrospira* post-harvest such that the *Arthrospira* enter the anabiotic state. In fact, as mentioned before, commercial growers of *Arthrospira* tend to minimise stressing of the organism as much as possible as the thick mucoid coating tends to clog machinery for processing the organism.

[0072] *Arthrospira* (Spirulina) powders were obtained from the following suppliers:

1. 1. China Spirulina - Jiangsu Cibainian Nutrition Food Co., Ltd. China
2. 2. Febico - Far East Biotech Co. Ltd. Taiwan
3. 3. Life Stream/Earthrise - DIC. California USA
4. 4. Pacifica - Cyanotech. Hawaii USA
5. 5. Siam Algae Co. - DIC. Thailand
6. 6. Spirin -Yunnan Spirin Co. Ltd. China
7. 7. Synergy - DIC. China

[0073] Each powder was mixed in an aqueous cream using 12.5% w/w concentration. Each cream was then decolourised. A uniform lawn of *T. rubrum* was grown on PDA medium in a dish. After 3-4 days of incubation, wells were cut in the PDA medium with a sterile cork borer. Measured amounts of each cream were placed in a respective well, the dishes were then incubated for 2-4 days.

[0074] Figure 4 shows the antifungal activities of 12.5% w/w Siam Algae Co. (3 o'clock position), 12.5% w/w Life Stream (6 o'clock position), and 12.5% w/w China Spirulina (9 o'clock position).

[0075] The results showed that all commercial powders tested had some antifungal activity. Respirometry confirmed that all commercial powders tested also had activity (presumably enzymatic) when incubated with chitin (results not shown).

[0076] One powder (Life Stream) was randomly selected for blending with an over-the-counter aqueous cream at a concentration of 12.5% w/w without further processing and tested for antifungal activity against *Aspergillus niger*. The cream produced a clearance of 4 mm, as seen in Figure 5 at the 12, 3, 6 and 9 o'clock positions. This indicated that possibly all commercial preparations of dried *Arthrospira* were likely to have some level of antifungal activity, without requiring an additional stressing step or further processing step such as filament disruption.

Example 6 - Activity of Stressed *Arthrospira* Against Fungal Diseases of Mammals

[0077] The antifungal activity of AMYCOT® was *tested in vitro* against different fungal diseases of mammals. The following pathogens were sourced from the Department of Primary Industries, Department of Agriculture, ACM and nurseries:

- *Candida albicans*
- *Microsporum canis*
- *Trichophyton mentagrophytes*
- *Trichophyton rubrum*
- *Epidermophyton floccosum*
- *Fusarium graminearum*
- *Alternaria sp.*
- *Malassezia furfur*

[0078] A uniform lawn of each pathogen was grown on PDA medium. After 1-2 days of incubation, wells were cut in the PDA medium with a sterile 8 mm diameter cork borer.

Measured amounts of AMYCOT® were placed in each well. The plates were cultured at an appropriate temperature depending on the pathogen. The welled plates were inspected daily. The clearance was measured after 2-3 days. Measurement of the zone is taken from the perimeter of the well to the outside perimeter of the zone.

[0079] This zone was void of live fungal cells proving efficacy of AMYCOT® against the target pathogens.

[0080] 12.5% w/w AMYCOT® cream produced a clearance of 8 mm of the pathogen *T. rubrum*. *T. rubrum* is a causal agent of hoof infections and ring worm.

[0081] 12.5% w/w AMYCOT® cream produced a clearance of 15 mm of the pathogen *T. mentagrophytes*. *T. mentagrophytes* is a causal agent of hoof infections and ring worm.

[0082] Figure 6 shows a well of 12.5% w/w AMYCOT® cream produced a clearance of 3 mm of the pathogen, *M. canis*. *M. canis* is a causal agent of hoof infections and ring worm.

[0083] 12.5% w/w AMYCOT® cream produced a clearance of 3 mm of the pathogen E. *floccosum*. *E. floccosum* is a casual agent of hoof infections and ring worm.

[0084] 12.5% w/w AMYCOT® cream produced a clearance of 4.5 mm of the pathogen *C. albicans*. *C. albicans* is a causal agent of ring worm and dermatitis.

[0085] Figure 7 shows a well of 12.5% w/w AMYCOT® cream produced a clearance of 12 mm of the pathogen *F. graminearum*. *F. graminearum* is a causal agent of hoof infections.

[0086] Figure 8 shows that wells of 12.5% w/w AMYCOT® cream produced clearances of *M. furfur*. *M. furfur* is a causal agent of dandruff.

[0087] Figure 9a shows a well of 5% w/w AMYCOT® shampoo produced a clearance of 3 mm of the pathogen *T. rubrum*.

[0088] Figure 9b shows a well of 5% w/w AMYCOT® shampoo produced a clearance of 4 mm of the pathogen *C. albicans*.

Example 7 - Activity of Stressed *Arthrospira* Against Fungal Diseases of Plants

[0089] The antifungal activity of AMYCOT® was *tested in vitro* against different fungal diseases of plants. The following pathogens were sourced from the Department of Primary Industries, Department of Agriculture, ACM and nurseries:

- *Fusarium graminearum*
- *Pithomyces Chartarum*

- *Botrytis cinerea*
- *Alternaria sp.*

[0090] A uniform lawn of each pathogen was grown on PDA medium. After 1-2 days of incubation, wells were cut in the PDA medium with a sterile 8 mm diameter cork borer. Measured amounts of AMYCOT® were placed in each well. The plates were cultured at an appropriate temperature depending on the pathogen. The welled plates were inspected daily.

[0091] The clearance was measured after 2-3 days. Measurement of the zone was taken from the perimeter of the well to the outside perimeter of the zone.

[0092] The zone was void of live fungal cells proving efficacy of AMYCOT® against the target pathogens.

[0093] 12.5% w/w AMYCOT® cream produced a clearance of 13 mm of the pathogen *F. graminearum*. *F. graminearum* is a causal agent of head blight and crown rot in small grain.

[0094] Figure 10 shows a well of 12.5% w/w AMYCOT® cream produced a clearance of 10 mm of the pathogen *P. chartarum*. *P. chartarum* is a causal agent of facial eczema in sheep cattle.

[0095] 12.5% w/w AMYCOT® cream produced a clearance of 8 mm of the pathogen *B. cinerea*. *B. cinerea* is a causal agent of botrytis in fruit.

[0096] 12.5% w/w AMYCOT® cream produced a clearance of 9 mm of the pathogen *Alternaria sp,* the casual agent of leaf spot in fruit.

Example 8 - Testing of AMYCOT® as a Spray on Plant Fungal Pathogens

[0097] AMYCOT® in the form of an antifungal spray was *tested in vitro* against a common plant pathogen, *Botrytis cinerea.* 5% w/w AMYCOT® powder was hydrated with water, but was only sparingly soluble.

[0098] A uniform lawn of *B. cinerea* was grown on PDA medium in a dish. The right-hand side of the dish was then sprayed with the spray and allowed to sit at room temperature for 24 hours. The left-hand side of the dish was not sprayed. Figure 11 shows that the sprayed side of the dish had dead fungi. The spray was effective in eliminating botrytis and inhibiting further sporulation.

Example 9 - *In Vitro* Antimicrobial Tests with Stressed *Arthrospira* and Monographed Actives

[0099] The antifungal and antibacterial activities of various over-the-counter fungal and antibacterial creams (including Lamisil™, Dakta Gold™, Canesten™, Tinaderm™, Daktarin™, Tripod Labs™, Resolve Tinea™, Resolve Balm™, Resolve Plus™, Clearasil™, Benzac W™) and AMYCOT® were tested in this and other examples.

[0100] In this example, the antifungal and antibacterial activities of various over-the-counter creams and AMYCOT® were tested by an independent laboratory (ConMac Laboratory Services) against the target pathogens *Trichophyton mentagrophytes, Epidermophyton floccosum, Trichophyton rubrum and Propionibacterium acnes*.

[0101] The results are shown in Table 6.

[0102] The results show that only AMYCOT® was active against each pathogen. The only

Table 6

Target Pathogen	Test Cream	Clearance Day 1	Clearance Day 2	Clearance Day 3
Trichophyton mentagrophytes	1) Control	Nil detected	Nil detected	Nil detected
	2) Amycot®	0.5-1 mm not clear, round	4 mm not clear, round	1-2 mm clear, round 3-4 mm not clear, round
	3) Lamisil	Nil detected	Nil detected	Nil detected
	4) Dakta Gold	Nil detected	Nil detected	Nil detected
Epidermophyton floccosum	1) Control	Nil detected	Nil detected	Nil detected
	2) Amycot®	1 mm clear, round	2.5 mm clear, round	2.5 mm clear, round 5 mm not clear, round
	3) Canesten	Nil detected	Nil detected	Nil detected
	4) Lamisil	Nil detected	Nil detected	Nil detected
Trichophyton rubrum	1) Control	Nil detected	Nil detected	Nil detected
	2) Amycot®	1 mm clear, round	5.5 mm clear, round	5.5 mm clear, round 4 mm not clear, round
	3) Tinaderm	Nil detected	Nil detected	Nil detected

Table 6				
Target Pathogen	**Test Cream**	**Clearance Day 1**	**Clearance Day 2**	**Clearance Day 3**
	4) Lamisil	Nil detected	1 mm clear, irregular	1.5 mm clear, irregular
Propionibacterium acnes	1) Control	Nil detected	Nil detected	Nil detected
	2) Amycot®	Nil detected	Nil detected	Nil detected
	3) Clearasil	Nil detected	Nil detected	Nil detected
	4) Benzac W	Nil detected	Nil detected	Nil detected

other instance of a cream showing activity was Lamisil™ against *T. rubrum.*

Example *10 -In Vitro* Tests with Monographed Actives and Combinations with AMYCOT®

[0103] Antifungals such as miconazole, tolnaftate, bifonazole and clotrimazole work by inhibiting specific protein production, preventing normal functions such as reproduction. The antifungal terbinafine works by preventing the formation of vital sterols.

[0104] The first aim of these *in vitro* tests was to determine the efficacies of various over-the-counter creams or their actives as fungal inhibitors and to compare the efficacy of AMYCOT®. The second aim was to determine the efficacy of AMYCOT® in combination with one or more of the over-the-counter creams or their actives as fungal inhibitors.

[0105] Sabouraud's liquid medium was inoculated with the chosen pathogen. The broths were then incubated for one week at 28°C. A sterile swab was dipped into a broth of the pathogen, and a PDA medium plate was inoculated by streaking the plate at right angles, insuring that the plate had an even lawn.

[0106] Monographed actives not already in a cream base were mixed with a respective cream base. A select cream was placed onto a 10 mm diameter filter paper disc and the disc was placed onto a newly inoculated plate. Up to four different discs were placed on a plate. The plate was then incubated for 24-48 hours at the appropriate temperature depending on the pathogen. Photos were then taken at the completion of the 24-48 hours.

[0107] The inhibitory test results are shown in Figures 12-21 and in Tables 7-16 below.

Table 7 - Inhibitory Test (Figure 12)		
Pathogen: *Trichophyton rubrum*		
Position	**Agent**	**Size of Clearance (mm)**
12:00	AMYCOT® 12.5% cream	8
3:00	Tinaderm (tolnaftate 2%)	0
6:00	Placebo	0
9:00	Daktarin (miconazole 2%)	15

Table 8 - Inhibitory Test (Figure 13)		
Pathogen: *Trichophyton mentagrophytes*		
Position	**Agent**	**Size of Clearance (mm)**
12:00	AMYCOT® 12.5% cream	4
3:00	Tinaderm (tolnaftate 2%)	1
6:00	Placebo	0
9:00	Daktarin (miconazole 2%)	11

Table 9 - Inhibitory Test (Figure 14)		
Pathogen: *Candida albicans*		
Position	**Agent**	**Size of Clearance (mm)**
12:00	AMYCOT® 12.5% cream	2
3:00	Tinaderm (tolnaftate 2%)	3
6:00	Placebo	0
9:00	Daktarin (miconazole 2%)	15

Table 10 - Inhibitory Test (Figure 15)	
Pathogen: *Candida albicans*	
Agent	**Size of Clearance (mm)**
Tripod Labs (clotrimazole 1% + tea tree oil)	10

Table 11 - Inhibitory Test (Figure 16)		
Pathogen: *Trichophyton mentagrophytes*		
Position	**Agent**	**Size of Clearance (mm)**
12:00	50% AMYCOT® 12.5% cream + 50% Daktarin (miconazole 2%)	10
3:00	Resolve Tinea (miconazole 2%)	10
6:00	Resolve Balm (miconazole 2%)	7
9:00	Resolve Plus (miconazole 2%)	11

Table 12 - Inhibitory Test (Figure 17)		
Pathogen: *Candida albicans*		
Position	**Agent**	**Size of Clearance (mm)**
12:00	AMYCOT® + Daktarin	10
3:00	Resolve Tinea (miconazole 2%)	6
6:00	Resolve Balm (miconazole 2%)	7
9:00	Resolve Plus (miconazole 2%)	11

Table 13 - Inhibitory Test (Figure 18)		
Pathogen: *Trichophyton mentagrophytes*		
Position	**Agent**	**Size of Clearance (mm)**
12:00	AMYCOT® 12.5% cream	1
3:00	AMYCOT 12% + clotrimazole 10%	13
6:00	AMYCOT 12% + terbinafine 1%	1
9:00	AMYCOT 12% + tolnaftate 10%	

Table 14 - Inhibitory Test (Figure 19)		
Pathogen: *Trichophyton mentagrophytes*		
Position	**Agent**	**Size of Clearance (mm)**
12:00	AMYCOT® 12.5% cream	1
3:00	AMYCOT 12% + clotrimazole 10%	11
6:00	AMYCOT 12% + terbinafine 1%	2
9:00	AMYCOT 12% + tolnaftate 10%	4

Table 15 - Inhibitory Test (Figure 20)		
Pathogen: *Trichophyton mentagrophytes*		
Position	**Agent**	**Size of Clearance (mm)**
12:00	AMYCOT® 12.5% cream + miconazole 2%	11
3:00	Placebo	0
6:00	Resolve Balm (miconazole 2%)	7
9:00	Resolve Plus (miconazole 2%)	7

Table 16 - Inhibitory Test (Figure 21)		
Pathogen: *Candida albicans*		
Position	**Agent**	**Size of Clearance (mm)**
12:00	50% AMYCOT® 12.5% cream + 50% Daktarin (miconazole 2%)	14
3:00	1% clotrimazole	4

Table 16 - Inhibitory Test (Figure 21)		
Pathogen: *Candida albicans*		
Position	**Agent**	**Size of Clearance (mm)**
6:00	10% tolnaftate	0
9:00	2% miconazole	9

[0108] The monographed actives in Figure 21 are in an aqueous cream base as opposed to a spirit base which is normally used.

[0109] In the *in vitro* tests miconazole and clotrimazole were seen as being the most effective inhibitors of pathological fungi, (see Figures 12-21). Figures 16 and 17 and Tables 11 and 12 establish that miconazole is an effective fungal inhibitor. AMYCOT® also acts as an inhibitor, but is only approximately 40% as effective as clotrimazole and miconazole (see Figures 12-14). Figures 18-21 and Tables 13-16 suggest that the combinations of AMYCOT® plus miconazole and AMYCOT® plus clotrimazole are the most effective fungal inhibitors *in vitro* when compared to terbinafine and tolnaftate.

[0110] The third aim of these *in vitro* tests was to determine the efficacies of various over-the-counter creams or their actives as fungicides and to compare the efficacy of AMYCOT®. The fourth aim was to determine the efficacy of AMYCOT® in combination with monographed actives.

[0111] The fungicidal test involved growing a lawn of the pathogen on PDA medium plates over a 2-3 day period and then exposing the mature pathogen to the antifungals. This was done by cutting wells into the lawned agar, and removing the agar and filling the wells with cream.

[0112] The fungicidal test results are shown in Figures 22-28 and Tables 17-23 below.

Table 17 - Fungicidal Test (Figure 22)		
Pathogen: *Trichophyton mentagrophytes*		
Position	**Agent**	**Size of Clearance (mm)**
12:00	AMYCOT® 12.5% cream	6
3:00	AMYCOT® 12.5% cream + 1% terbinafine	6
6:00	AMYCOT® 12.5% cream + 10% tolnaftate	6
9:00	AMYCOT® 12.5% cream + 10% clotrimazole	5

Table 18 - Fungicidal Test (Figure 23)		
Pathogen: ***Trichophyton rubrum***		
Position	**Agent**	**Size of Clearance (mm)**
12:00	AMYCOT® 12.5% cream	9
3:00	AMYCOT® 12.5% cream + 10% clotrimazole	7
6:00	AMYCOT® 12.5% cream + 1% miconazole	8
9:00	Placebo	0

Table 19 - Fungicidal Test (Figure 24)		
Pathogen: ***Trichophyton mentagrophytes***		
Position	**Agent**	**Size of Clearance (mm)**
12:00	AMYCOT® 12.5% cream	6
3:00	AMYCOT® 12.5% cream + 1% terbinafine	5
6:00	AMYCOT® 12.5% cream + 10% tolnaftate	5
9:00	AMYCOT® 12.5% cream + 10% clotrimazole	4

Table 20 - Fungicidal Test (Figure 25)		
Pathogen: ***Trichophyton mentagrophytes***		
Position	**Agent**	**Size of Clearance** (mm)
12:00	AMYCOT® 12.5% cream	8
3:00	AMYCOT® 12.5% cream + 1% terbinafine	7
6:00	AMYCOT® 12.5% cream + 10% tolnaftate	5
9:00	AMYCOT® 12.5% cream + 10% clotrimazole	4

Table 21 - Fungicidal Test (Figure 26)		
Pathogen: ***Trichophyton mentagrophytes***		
Position	**Agent**	**Size of Clearance** (mm)
12:00	AMYCOT® 12.5% cream	9
3:00	AMYCOT® 12.5% cream + 1% terbinafine	4
6:00	AMYCOT® 12.5% cream + 10% tolnaftate	5
9:00	AMYCOT® 12.5% cream + 10% clotrimazole	5

Table 22 - Fungicidal Test (Figure 27)		
Pathogen: *Candida albicans*		
Position	**Agent**	**Size of Clearance (mm)**
12:00	AMYCOT® 12.5% cream	8
3:00	Daktarin (miconazole 2%)	0
6:00	Canesten (clotrimazole 1 %)	0
9:00	AMYCOT® 12% + clotrimazole 1%	5

Table 23 - Fungicidal Test (Figure 28)		
Pathogen: *Trichophyton rubrum*		
Position	**Agent**	**Size of Clearance** (mm)
12:00	AMYCOT® 12.5% cream	6
3:00	10% clotrimazole	0
6:00	10% tolnaftate	0
9:00	2% miconazole	0

[0113] The monographed actives in Figure 28 are in an aqueous cream base as opposed to a spirit base which is normally used.

[0114] The results support that the present over-the-counter antifungals and monographed actives are fungi-static agents and not fungicides. Figures 22-28 and Tables 17-23 demonstrate that AMYCOT® is an efficacious fungicide, even when combined with monographed actives.

[0115] The combination of AMYCOT® with either miconazole or clotrimazole did not greatly reduce the efficacies of either. The combination increased the inhibitory effect (see Figures 17-21). This created a very effective broad spectrum product.

[0116] The combination of AMYCOT® with clotrimazole slightly inhibited the antifungal activity of AMYCOT® (see Figures 22-28), but the combination of AMYCOT® with miconazole had no great detrimental effects on the inhibitory or the fungicidal activity of the combination cream (see Figures 17-26).

[0117] The combination cream of AMYCOT® and miconazole was more efficacious as a broad spectrum product than either due to the inhibitory nature of miconazole and both the inhibitory and fungicidal activities of AMYCOT®.

[0118] The combination cream acted as an inhibitor and then "held its ground", while clearances achieved with miconazole alone became encroached by regrowth as in the case of the pathogen *T. mentagrophytes* (see Figure 20).

[0119] It was also found that the combination cream AMYCOT® and 2% miconazole restricted regrowth of the pathogen after a clearance was made around the wells.

Example 11 - Trials of *Arthrospira* on Various Skin Conditions

[0120] A subject had advanced athlete's foot, with deep lesions between the toes. The subject reported that itching caused by the fungus stopped within 15 minutes of first applying 12.5% w/w AMYCOT® cream. Inflammation reduced and in some cases disappeared completely in 12 hours. The lesions began closing within 24 hours and completely healed within 4-5 days.

[0121] These observations were repeated in the case of acne with subjects recording that the associated inflammation disappeared and that their skin returned to normal colour and became soft and pliable like new skin. The pustules also dried out.

[0122] It is the belief of the inventors that one or more biocidal agents (eg. chitinase, chitosanase, chitin deacetylase) interact synergistically with other components of *Arthrospira* to give rise to the therapeutic effects. The itching in fungal infections is an indication of the release of digestive enzymes or metabolites from the fungi. Once normal fungal metabolism is disrupted as the cell wall is disrupted, itching ceases.

[0123] Similar results were obtained when using the cream for conditions unrelated to fungus, on dry and cracked heels and elbows, rosacea, eczema, sun damaged skin and psoriasis. Subjects reported almost immediate relief from itching in dermatitis.

Example 12 - Antifungal Activity of Stressed *Arthrospira* Using "Unprocessed" Powder

[0124] Example 5 supported that commercial preparations of dried *Arthrospira* were likely to have some level of antifungal activity without requiring an additional stressing step or post-harvest processing step such as filament disruption. Example 12 further supports this finding.

[0125] A dried preparation of *Arthrospira maxima* was sourced from a commercial grower. The preparation was blended with an over-the-counter aqueous cream at a concentration of 12.5% w/w without further processing, and was tested for antifungal activity against *T. mentagrophytes, T. rubrum* and *M. fructicola* (as described in Example 5).

[0126] As seen in Figure 31, the cream produced a clearance against *T. mentagrophytes* at the 12, 3, 6 and 9 o'clock positions.

[0127] As seen in Figure 32, the cream produced a clearance against *T. rubrum* at the 6 o'clock position.

[0128] As seen in Figure 33, the cream produced a clearance against *M. fructicola* at the 6 o'clock position.

[0129] These findings support that post-harvest steps such as explosive decompression, dry freezing, decolourisation and milling need not be carried out in order to produce *Arthrospira* having fungicidal activity.

Example 13 - Antifungal Activity of *Arthrospira* Not Explosively Decompressed

[0130] This example further supports that the post-harvesting step of explosive decompression (potentiation) is not required in order to produce *Arthrospira* having fungicidal activity.

[0131] A dry preparation of *Arthrospira maxima* was sourced from a commercial grower. In one instance, dried *Arthrospira* was disrupted by explosive decompression (potentiated) using the method described in New Zealand Patents No. 328013 and No. 328740. The *Arthrospira* was re-hydrated, decolourised, dried and milled to form a dry "premix powder". The premix powder was then suspended in a suitable carrier (12.5%) and will be referred to as "potentiated AMYCOT®".

[0132] In another instance, the preparation excluded the explosive decompression method described in New Zealand Patents No. 328013 and No. 328740, and this preparation will be referred to as "original AMYCOT®".

[0133] As seen in Figure 34, both "original AMYCOT®" and "potentiated AMYCOT®" produced almost equal clearances against *Alternaria sp.* at the 12 and 7 o'clock positions, respectively. Hence, explosive decompression is not an essential post-harvest step for producing *Arthrospira* having fungicidal activity.

[0134] The term "comprise" and variants of the term such as "comprises" or "comprising" are used herein to denote the inclusion of a stated integer or stated integers but not to exclude any other integer or any other integers, unless in the context or usage an exclusive interpretation of the term is required.

[0135] Any reference to publications cited herein is not an admission that the disclosures constitute common general knowledge in Australia.

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in

compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- WO1998025471A [0003]
- NZ336620 [0021] [0026] [0029]
- NZ336619 [0021] [0026] [0029]
- NZ328013 [0026] [0045] [0131] [0132]
- NZ328740 [0026] [0045] [0131] [0132]

Non-patent literature cited in the description

- **J. F. MANENJ. FALQUET**The cpcB--cpcA locus as a tool for the genetic characterization of the genus Arthrospira (Cyanobacteria): evidence for horizontal transferInternational Journal of Systematic and Evolutionary Microbiology, 2002, vol. 52, 861-867 [0023]
- Ultrastructure and Taxonomy of Arthrospira (Spirulina) Maxima and Arthrospira (Spirulina) Platensis**BY LUISA TOMASELLI**Physiology, Cell-biology and BiotechnologyTaylor and Francis19970000 [0023]

Patentkrav

1. Fremgangsmåde til fremstilling af en biocidsammensætning til topisk anvendelse på et emne, hvilken fremgangsmåde omfatter trinene:
(1) fysiologisk stressning af Arthrospira ved mangel på næringsstoffer eller lys, ved dehydrering eller ved fjernelse af op til 80 % af væsken, hvori Arthrospira dyrkes; og
(2) kombinering af en biocidvirkende effektiv mængde af den stressede Arthrospira (1) med et bærematerialer, et opløsningsmiddel, en base eller et excipiens, der er egnet til topisk administration på emnet, hvor den biocidvirkende effektive mængde er effektiv mod bakterier op emnet eller huddefekter på et pattedyr; og
hvor Arthrospira ikke er blevet frysetørret.

2. Fremgangsmåde ifølge krav 1, hvor trinet fysiologisk stressning af Arthrospira omfatter fjernelse af op til 80 % af væsken, hvori Arthrospira dyrkes; og trin (2) forudgås af trinene (1a) og (1b) som følger:
(1a) vask af den stressede Arthrospira til fjernelse af kontaminanter; og
(1b) tørring af den vaskede Arthrospira.

3. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 2, hvor bærematerialet, opløsningsmidlet, basen eller excipienset, der er egnet til topisk administration på emnet er et bærematerialе, et opløsningsmiddel, en base eller et excipiens til topisk administration på et menneske, et dyr, et landbrugs- eller havebrugsprodukt, jord, en menneskeskabt struktur eller en vandvej.

4. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 3, hvor huddefekten er valgt fra gruppen, der består af akneskade, rosacea, et rødt område, en revne, et brandsår, en blist, psoriasis, eksem, skældannelse, rynker, stomatitis, en læsion, en pustel, et sår, arp, bleeksem, et ulcus, et forkølelsessår, skægpest, dermatitis og revnede hæle og

albuer.

5. Topisk biocidsammensætning, der omfatter en biocidvirkende effektiv mængde af fysiologisk stresset Arthrospira, hvor den fysiologiske stressning opnås ved mangel på næringsstoffer eller lys, ved dehydrering eller ved fjernelse af op til 80 % af væsken, hvori Arthrospira dyrkes, og et bæremateriale, et opløsningsmiddel, en base eller et excipiens, der er egnet til topisk administration på et emne, hvor den biocidvirkende effektive mængde er effektiv mod bakterier på emnet eller huddefekter på et pattedyr; og hvor Arthrospira ikke er frysetørret.

6. Topisk biocidsammensætning ifølge krav 5, hvor emnet er et menneske, et dyr, et landbrugs- eller havebrugsprodukt, jord, en menneskeskabt struktur eller en vandvej, og hvor pattedyret er et menneske.

7. Topisk biocidsammensætning ifølge krav 5 eller krav 6, hvor huddefekten er valgt fra gruppen, der består af akneskade, rosacea, et rødt område, en revne, et brandsår, en blist, psoriasis, eksem, skældannelse, rynker, stomatitis, en læsion, en pustel, et sår, arp, bleeksem, et ulcus, et forkølelsessår, skægpest, dermatitis og revnede hæle og albuer.

8. Biocidvirkende effektiv mængde af fysiologisk stresset Arthrospira, hvor den fysiologiske stressning opnås ved mangel på næringsstoffer eller lys, ved dehydrering eller ved fjernelse af op til 80 % af væsken, hvori Arthrospira dyrkes, og et bæremateriale, et opløsningsmiddel, en base eller et excipiens til topisk administration på et emne til anvendelse til forebyggelse eller behandling af en bakteriel infektion eller infestation på emnet eller til behandling eller forebyggelse af en huddefekt hos et pattedyr; hvor Arthrospira ikke er frysetørret.

9. Biocidvirkende effektiv mængde af ikke-frysetørret,

fysiologisk stresset Arthrospira til anvendelserne ifølge krav 8, hvor ved anvendelsen til forebyggelse eller behandling af en bakteriel infektion eller infestation på et emne, emnet er et menneske, et dyr, et landbrugs- eller havebrugsprodukt, jord, en menneskeskabt struktur eller en vandvej, og hvor ved anvendelsen til behandling eller forebyggelse af en huddefekt hos et pattedyr, pattedyret er et menneske.

10. Biocidvirkende effektiv mængde af ikke-frysetørret, fysiologisk stresset Arthrospira til anvendelserne ifølge krav 8 eller krav 9, hvor emnet er et menneske, og den bakterielle infektion er forårsaget af Propionibacterium acnes.

11. Biocidvirkende effektiv mængde af ikke-frysetørret, fysiologisk stresset Arthrospira til anvendelserne ifølge krav 8 eller krav 9 til behandling eller forebyggelse af en huddefekt hos et pattedyr, fortrinsvis et menneske, hvor huddefekten er valgt fra gruppen, der består af akneskade, rosacea, et rødt område, en revne, et brandsår, en blist, psoriasis, eksem, skældannelse, rynker, stomatitis, en læsion, en pustel, et sår, arp, bleeksem, et ulcus, et forkølelsessår, skægpest, dermatitis og revnede hæle og albuer.

12. Biocidvirkende effektiv mængde af ikke-frysetørret, fysiologisk stresset Arthrospira til anvendelserne ifølge et hvilket som helst af kravene 8 til 11, hvor Arthrospira er A. maxima.

13. Topisk biocidsammensætning, der omfatter en biocidvirkende effektiv mængde af ikke-frysetørret, fysiologisk stresset Arthrospira ifølge et hvilket som helst af kravene 5 til 7, hvor Arthrospira er A. maxima.

14. Fremgangsmåde til fremstilling af en biocidsammensætning ifølge et hvilket som helst af kravene 1 til 4, hvor Arthrospira er A. maxima.

DRAWINGS

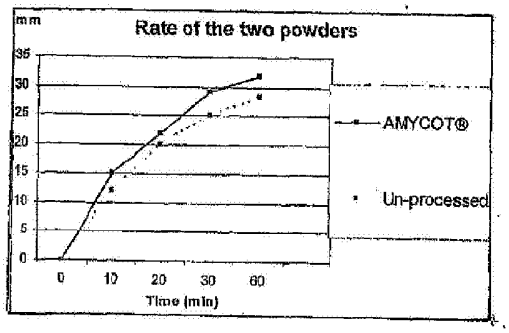


FIG. 1

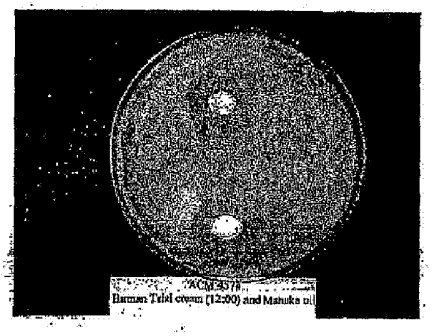


FIG. 2

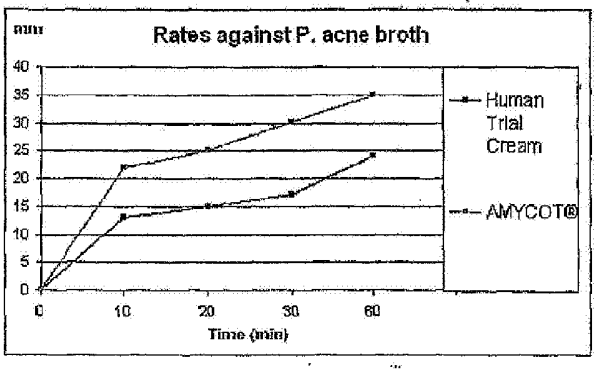


FIG. 3

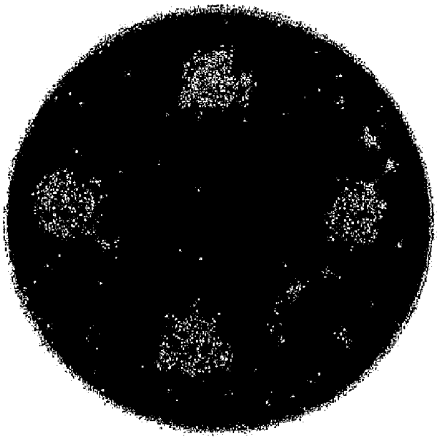


FIG. 5

Aspergillus niger

Life stream 12.5% original powder

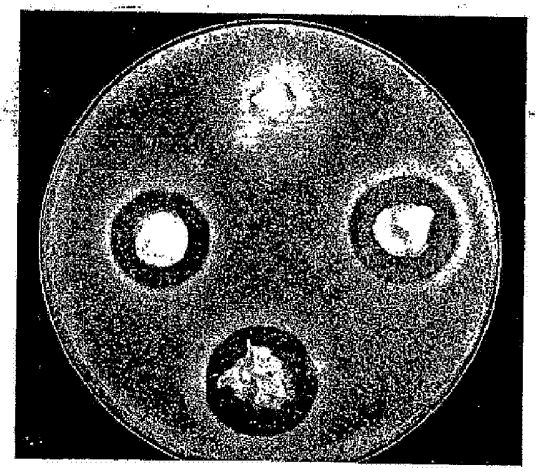


FIG. 4

T. rubrum

3:00 – Siam algae Co (12.5%)

6:00 – Life Stream (12.5%)

9:00 – China Spirulina (12.5%)

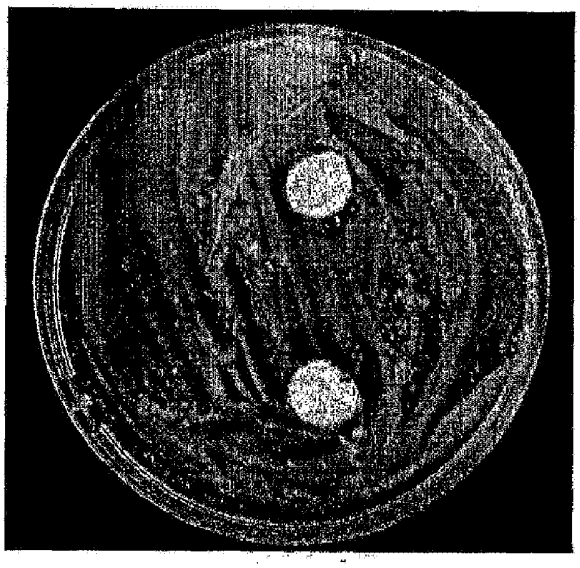


FIG. 8

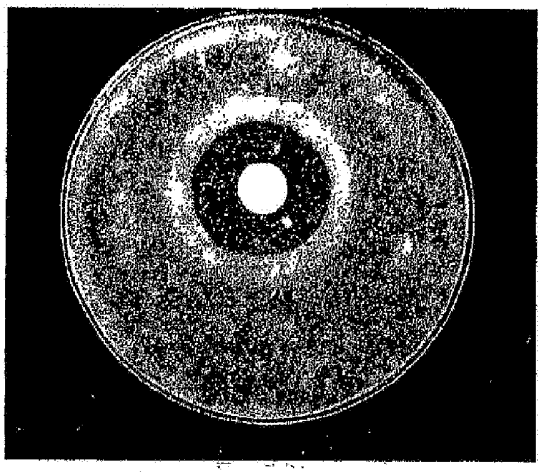


FIG. 7

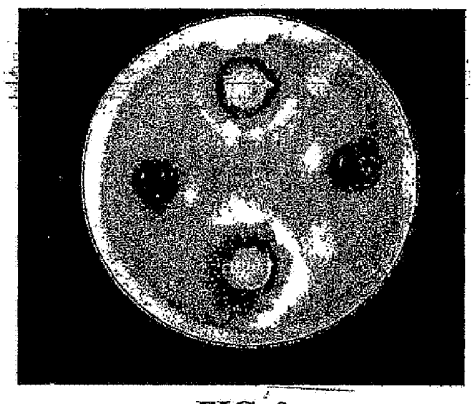


FIG. 9a

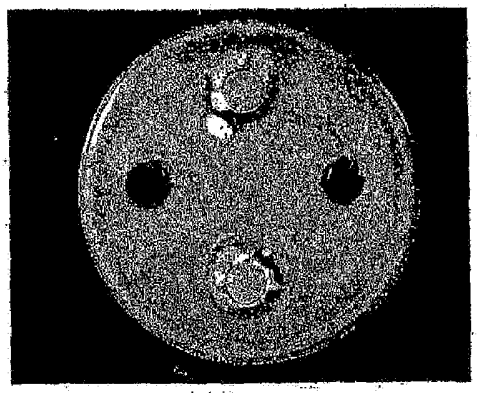


FIG. 9b

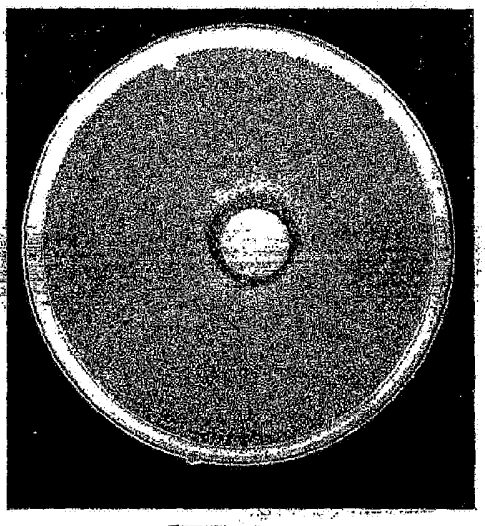


FIG. 6

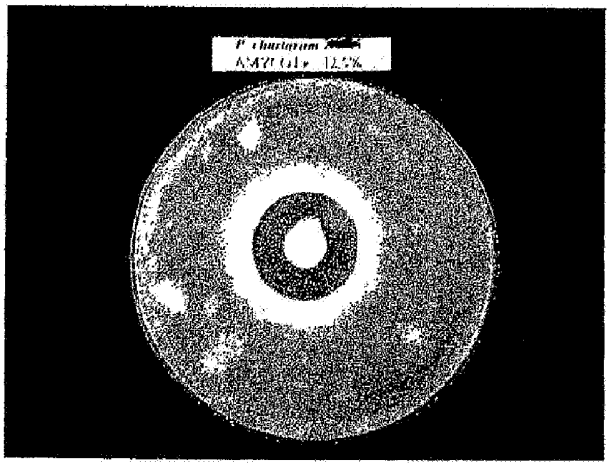


FIG. 10

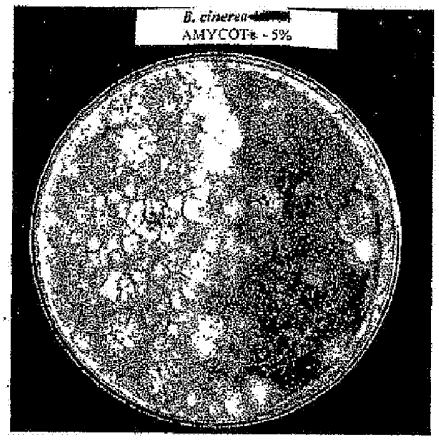


FIG. 11

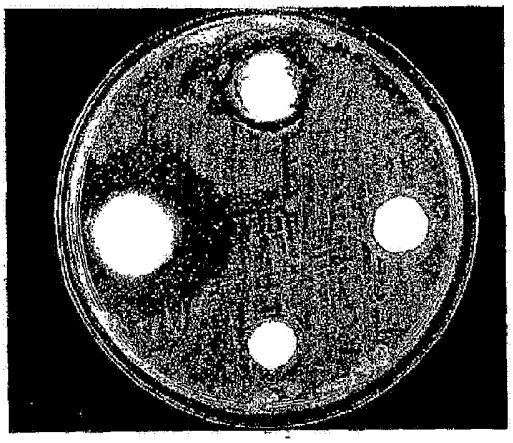


FIG. 13

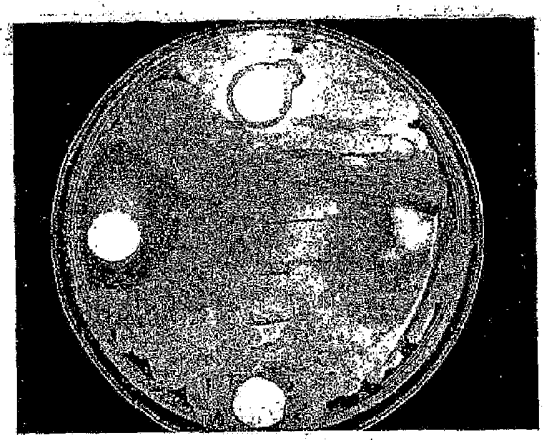


FIG. 14

Inhibitory Test
(Pathogen: Candida albicans)

12:00 - AMYCOT® 12.5% cream – 2mm
3:00 - Tinaderm (tolnaftate 2%) – 3mm
6:00 – placebo – 0mm
9:00 - Daktarin (miconazole 2%) – 15mm



FIG. 15

Inhibitory Test
(Pathogen: Candida albicans)

Tripod lab cream (clotrimazole 1% + tea tree oil) – 10mm

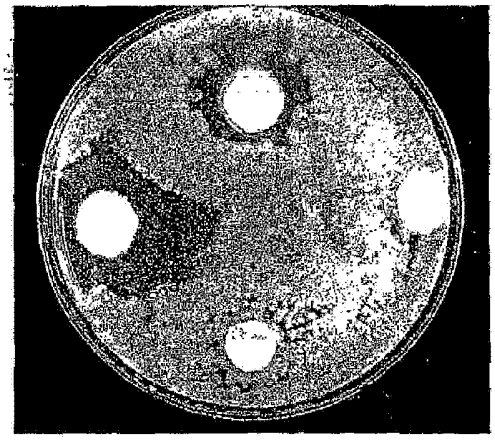


FIG. 12

Inhibitory Test
(Pathogen: Trichophyton rubrum)

12:00 - AMYCOT® 12.5% cream – 8mm
3:00 - Tinaderm (tolnaftate 2%) – 0mm
6:00 - Placebo – 0mm
9:00 - Daktarin (miconazole 2%) – 15mm

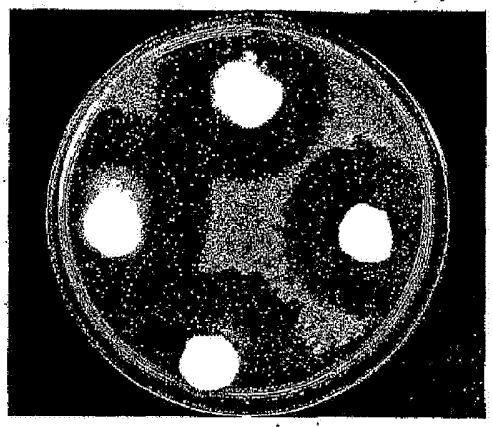


FIG. 16

Inhibitory Test
(Pathogen: Trichophyton mentagrophytes)

50% AMYCOT® 12.5% cream + 50% Daktarin (miconazole 2%) – 10mm
Resolve Tinea (miconazole 2%) – 10mm
Resolve Balm (miconazole 2%) – 7mm
Resolve Plus (miconazole 2%) – 11mm

(NOTE: combined cream is effectively 6% AMYCOT® + 1% miconazole)

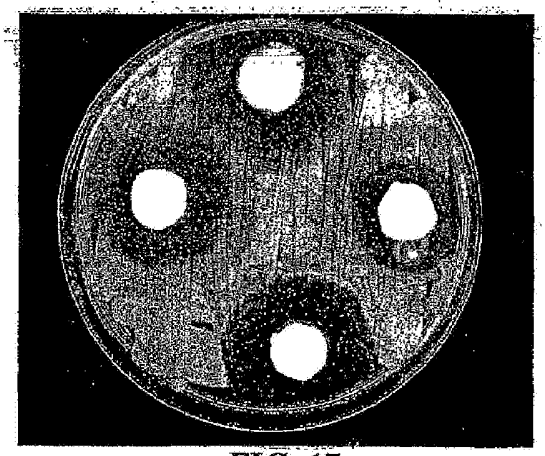


FIG. 17

(Pathogen: Candida albicans)

12:00 - AMYCOT® + Daktarin – 10mm
Resolve tinea (miconazole 2%) – 6mm
Resolve Balm (miconazole 2%) – 7mm
Resolve Plus (miconazole 2%) – 11mm

(NOTE: combined cream is effectively 6% AMYCOT® + 1% miconazole)

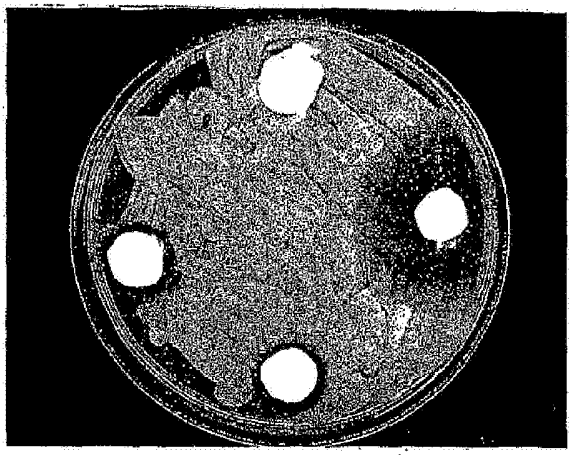


FIG. 18

Inhibitory Test
(Pathogen: Trichophyton mentagrophytes)

12:00 - AMYCOT® 12.5% cream – 1mm
3:00 - AMYCOT 12% + clotrimazole 10% – 13mm
6:00 - AMYCOT 12% + terbinafine1% – 1mm
9:00 - AMYCOT 12% + tolnaftate 10% – 1mm

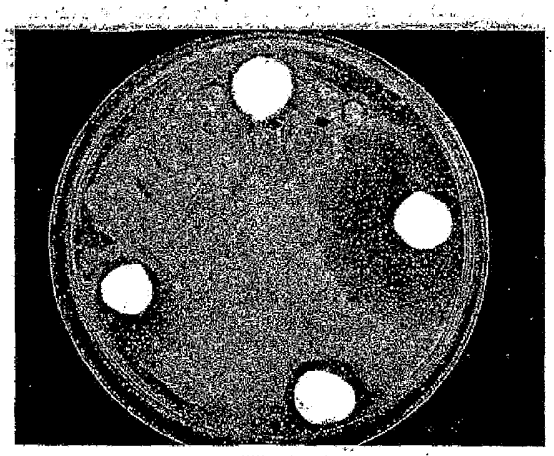


FIG. 19

Inhibitory Test
(Pathogen: Trichophyton mentagrophytes)

12:00 - AMYCOT® 12.5% cream – 1mm
3:00 - AMYCOT 12% + clotrimazole 10% – 11mm
6:00 - AMYCOT 12% + terbinafine1% – 2mm
9:00 - AMYCOT 12% + tolnaftate 10% – 4mm

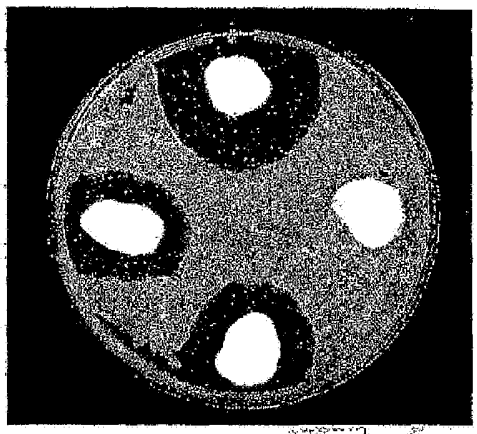


FIG. 20

Inhibitory Test
(Pathogen: Trichophyton mentagrophytes)

12:00 - AMYCOT® 12.5% cream + miconazole 2% – 11mm
3:00 – placebo – 0mm
6:00 -Resolve Balm (miconazole 2%) – 7mm
9:00 - Resolve Plus (miconazole 2%) – 7mm

(NOTE: combined cream is effectively 6% AMYCOT® + 1% miconazole)

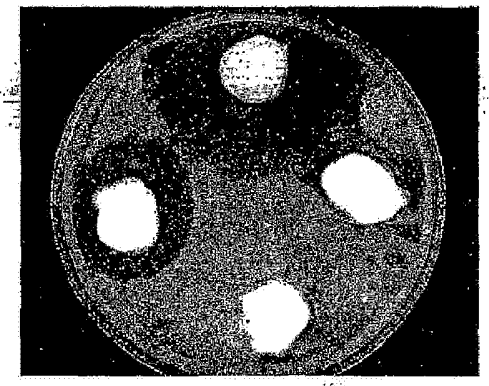


FIG. 21

Inhibitory Test
(Pathogen: Candida albicans)

12:00 - 50% AMYCOT® 12.5% cream + 50% Daktarin (miconazole 2%) – 14mm
3:00 – 1% clotrimazole – 4mm
6:00 -10% tolnaftate – 0mm
9:00 – 2%miconazole – 9mm

(NOTE: combined cream is effectively 6% AMYCOT® + 1% miconazole)

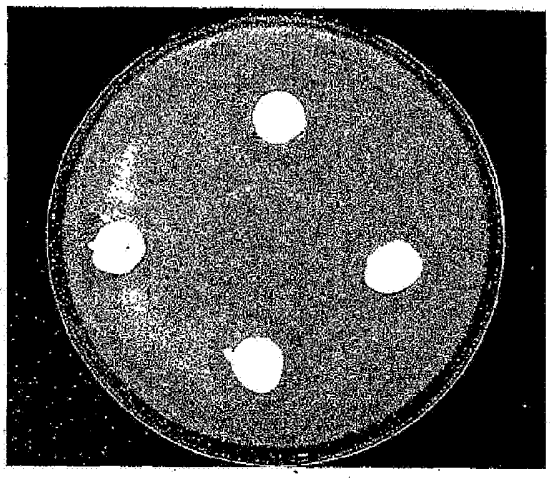


FIG. 22

Fungicidal Test
(Pathogen: Trichophyton mentagrophytes)

12:00 - AMYCOT® 12.5% cream – 6mm
3:00 - AMYCOT® 12.5% cream + 1% terbinafine – 6mm
6:00 - AMYCOT® 12.5% cream + 10% tolnaftate – 6mm
9:00 - AMYCOT® 12.5% cream + 10% clotrimazole – 5mm

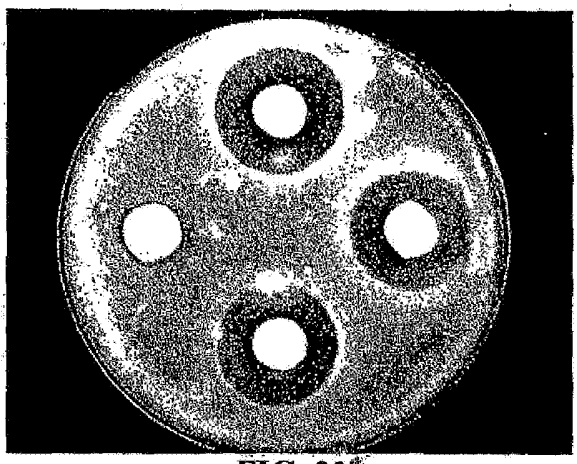


FIG. 23
Fungicidal Test
(Pathogen: Trichophyton rubrum)

12:00 - AMYCOT® 12.5% cream – 9mm
3:00 - AMYCOT® 12.5% cream + 10% clotrimazole – 7mm
6:00 - AMYCOT® 12.5% cream + 1% miconazole – 8mm
9:00 - placebo – 0mm

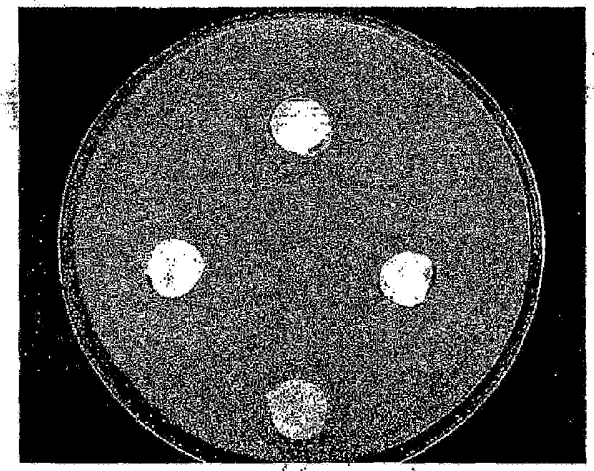


FIG. 24
Fungicidal Test
(Pathogen: Trichophyton mentagrophytes)

12:00 - AMYCOT® 12.5% cream – 6mm
3:00 - AMYCOT® 12.5% cream + 1% terbinafine – 5mm
6:00 - AMYCOT® 12.5% cream + 10% tolnaftate – 5mm
9:00 - AMYCOT® 12.5% cream + 10% clotrimazole – 4mm

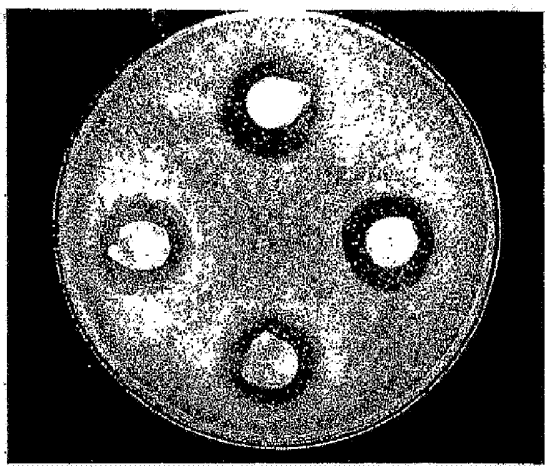


FIG. 25

Fungicidal Test
(Pathogen: Trichophyton mentagrophytes)

12:00 - AMYCOT® 12.5% cream – 8mm
3:00 - AMYCOT® 12.5% cream + 1% terbinafine – 7mm
6:00 - AMYCOT® 12.5% cream + 10% tolnaftate – 5mm
9:00 - AMYCOT® 12.5% cream + 10% clotrimazole – 4mm

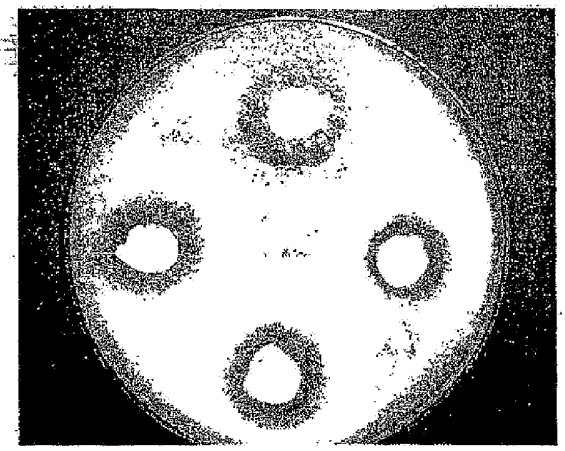


FIG. 26

Fungicidal Test
(Pathogen: Trichophyton mentagrophytes)

12:00 - AMYCOT® 12.5% cream – 9mm
3:00 - AMYCOT® 12.5% cream + 1% terbinafine – 4mm
6:00 - AMYCOT® 12.5% cream + 10% tolnaftate – 5mm
9:00 - AMYCOT® 12.5% cream + 10% clotrimazole – 5mm

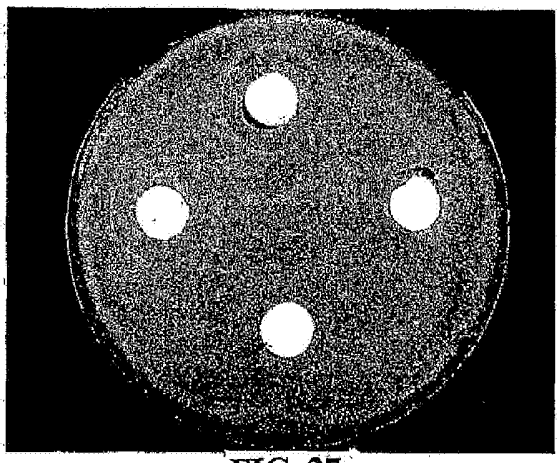


FIG. 27
Fungicidal Test
(Pathogen: Candida albicans)

12:00 - AMYCOT® 12.5% cream – 8mm
3:00 - Daktarin (miconazole 2 %) – 0mm
6:00 - Canesten (clotrimazole 1%) – 0mm
9:00 - AMYCOT® 12% + clotrimazole 1% – 5mm

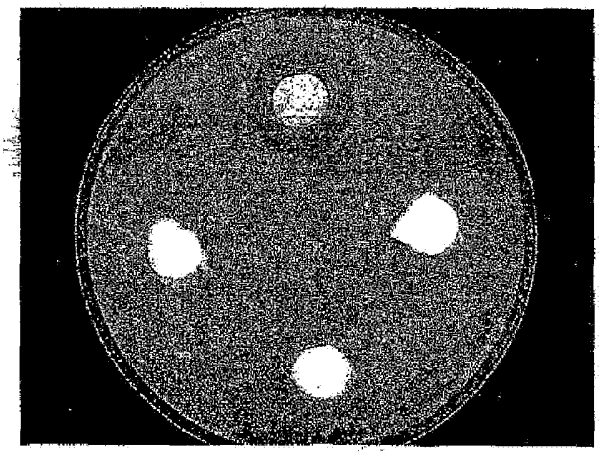


FIG. 28
Fungicidal Test
(Pathogen: Trichophyton rubrum)

12:00 - AMYCOT® 12.5% cream – 6mm
3:00 - 10% clotrimazole – 0mm
6:00 - 10% tolnaftate – 0mm
9:00 - 2% miconazole – 0mm

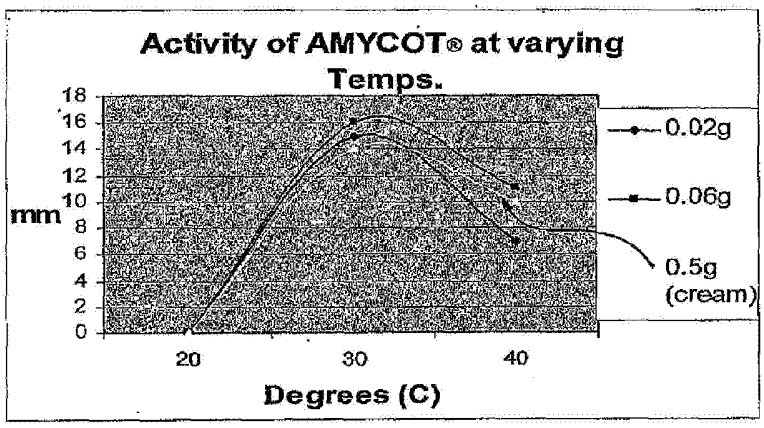

Activity of AMYCOT® at varying Temps.
mm
18
16
14
12
10
8
6
4
2
0
20
30
40
Degrees (C)
0.02g
0.06g
0.5g (cream)

FIG. 29

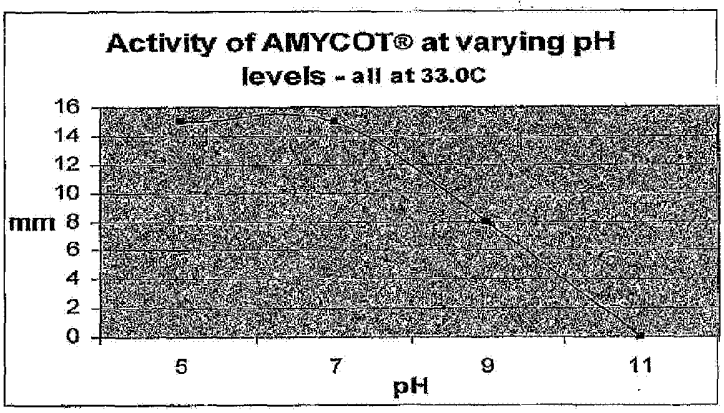

Activity of AMYCOT® at varying pH levels - all at 33.0C
mm
16
14
12
10
8
6
4
2
0
5
7
9
11
pH

FIG. 30

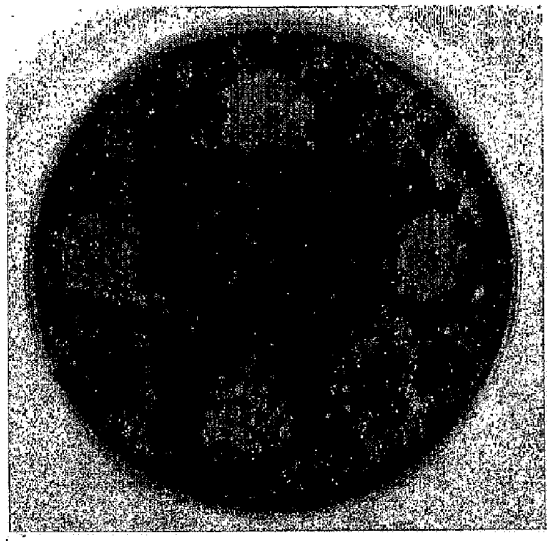


FIG. 31

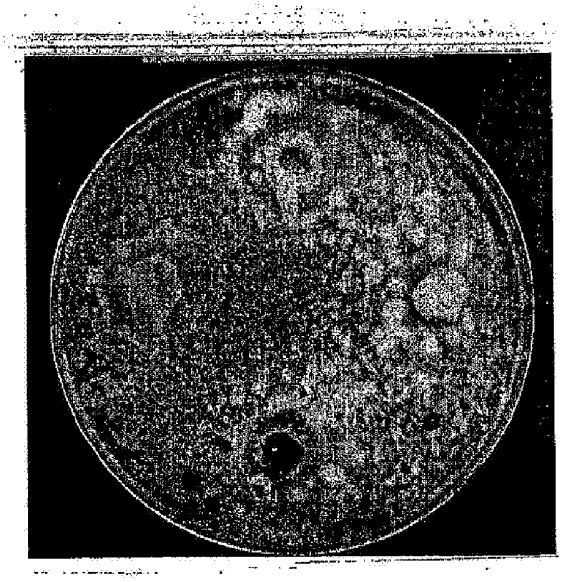


FIG. 32

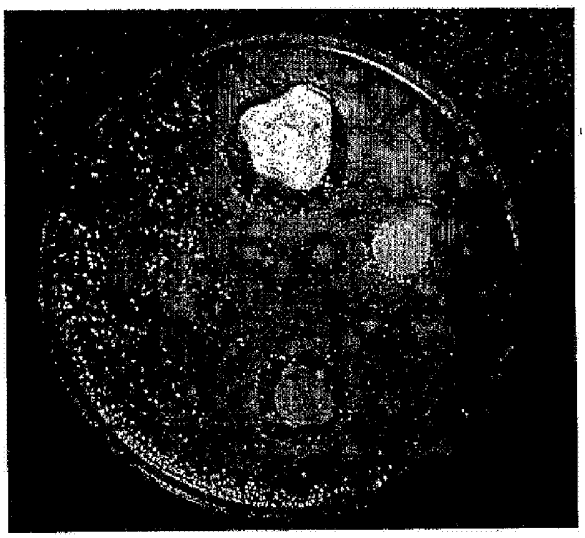


FIG. 33

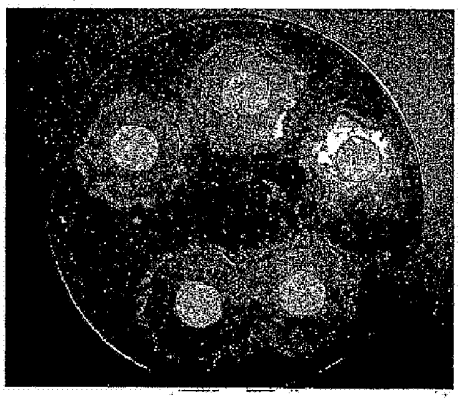


FIG. 34