

TITLE OF THE INVENTION

Arthrospira-based Compositions and Uses Thereof

TECHNICAL FIELD

The present invention relates to a composition comprising *Arthrospira* and the
5 use of the composition as a biocide and/or therapeutic.

BACKGROUND ART

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,
10 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>.

SUMMARY OF THE INVENTION

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

Physiologically Stressed *Arthrospira* has Biocidal Activity

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
20 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
25 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
30 bacteria is one such polymer, comprising N-acetylglucosamine and N-acetylmuramic acid.

According to a first aspect of the present invention, there is provided a
composition for digesting or modifying chitin, a chitin derivative or a polymer having
N-acetyl-D-glucosamine as a polymer subunit, said composition comprising
physiologically stressed *Arthrospira*.

According to a second aspect of the present invention, there is provided a method for digesting or modifying chitin, a chitin derivative or a polymer having N-acetyl-D-glucosamine as a polymer subunit, said method comprising the step of contacting chitin, chitin derivative or a polymer having N-acetyl-D-glucosamine as a polymer subunit with a composition comprising physiologically stressed *Arthrospira*.

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.

According to a third aspect of the present invention, there is provided a method for identifying a bioactive agent from *Arthrospira*, said method comprising the steps of:

(I) combining a composition comprising physiologically stressed *Arthrospira* with at least one test substrate modifiable by a bioactive agent from *Arthrospira*; and

(II) assaying for modification of the test substrate.

Any suitable type of test substrate can be used. Assaying for modification of the test substrate can be carried out in any suitable way. High throughput screening, however, is preferred.

Preferably, the bioactive agent is 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.

The method can further comprise the step of purifying the bioactive agent. Purification can be carried out in any suitable way. The method can also comprise the steps of cloning and expressing the gene/s of the bioactive agent. These steps can be carried out in any suitable way. For instance, the steps can be as described in Maniatis, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982, the entire contents of which are incorporated herein by cross reference.

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

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*).

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.

According to a fourth aspect of the present invention, there is provided a composition for use as a biocide and/or therapeutic, said composition comprising an effective amount of physiologically stressed *Arthrospira*.

According to a fifth aspect of the present invention, there is provided a method for preventing or treating an infection or infestation of a subject by an organism, said method comprising the step of administering to the subject an effective amount of a composition comprising physiologically stressed *Arthrospira*.

Preferably, the method comprises the step of identifying a subject in need of such treatment or requiring such prevention.

There is also provided the use of physiologically stressed *Arthrospira* in the preparation of a medicament for the prevention or treatment of an infection or infestation of a subject by an organism.

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.

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.

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.

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.

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*.

Some of the above steps are described, for example, in the specifications of New Zealand Patents No. 336620 and 336619, the entire contents of which are incorporated herein by cross reference. 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, the entire contents of which are incorporated herein by cross
5 reference.

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,
10 fragrance, colourant and preservative.

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.

15 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
20 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.

For therapeutic use, the composition can be administered or applied, for example, orally, parenterally, by inhalation spray, topically, rectally, nasally, buccally or vaginally.
25 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.

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

According to a sixth aspect of the present invention, there is provided a method for preparing an *Arthrospira*-based composition for use as a biocide and/or therapeutic, said method comprising the steps of:

(1) physiologically stressing *Arthrospira*; and

(2) combining the stressed *Arthrospira* of (1) with a suitable carrier, solvent, base or excipient.

According to a seventh aspect of the present invention, there is provided a method for preparing an *Arthrospira*-based composition for use as a biocide and/or therapeutic, said

5 method comprising the steps:

(1) physiologically stressing *Arthrospira* by removing up to about 80% of liquid within which the *Arthrospira* is being grown;

(2) washing the stressed *Arthrospira* in order to remove contaminants;

(3) drying the washed *Arthrospira*; and

10 (4) combining the washed *Arthrospira* with a suitable carrier, solvent, base or excipient.

Either of the above methods can be used to prepare one or more of the compositions described herein.

Preferably, the above methods exclude specific steps of explosive decompression and/or freeze drying the physiologically stressing *Arthrospira* prior to adding the carrier,

15 solvent, base or excipient.

Use of Physiologically Stressed *Arthrospira* as an Antifungal

According to an eighth aspect of the present invention, there is provided a composition for use as an antifungal, said composition comprising an effective amount of physiologically stressed *Arthrospira*.

20 According to a ninth aspect of the present invention, there is provided a method for preventing or treating a fungal infection or infestation of a subject, said method comprising the step of administering to the subject an effective amount of a composition comprising physiologically stressed *Arthrospira*.

Preferably, the method comprises the step of identifying a subject in need of
25 such treatment or requiring such prevention.

There is also provided the use of physiologically stressed *Arthrospira* in the preparation of a medicament for the prevention or treatment of a fungal infection or infestation of a subject.

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

In a first embodiment, the subject is a human or an animal such as, mammal or vertebrate. In human subjects, the composition can be used to treat:

- tinea (e.g. athlete's foot, jock itch, ringworm, deep nail bed infection) which is caused chiefly by species of *Microsporum*, *Trichophyton*, *Candida* and *Epidermophyton*;
- thrush, which is caused by *Candida*;
- 5 - dandruff, which is caused by *Malassezia*;
- tropical mycetoma - a granular spreading growth in the skin, muscle tissue and lymph nodes - which is caused by *Madurella*;
- sporothricosis - a granular spreading growth in skin and lymph nodes - which is caused by *Sporothrix schenckii*; and
- 10 - histoplasmosis - a chronic pneumonia with spreading systemic infection - which is caused by *Histoplasma capsulatum*.

In animal subjects, the composition can be used to treat, for example, ring worm, hoof infections (*Candida*, *Malassezia*), dermatitis and folliculitis (*Microsporum*, *Trichophyton*, *Alternaria*, *Fusarium*). Topical fungal conditions in animals are usually
15 caused by the same fungi as in humans.

In a second embodiment, the subject is an agricultural or horticultural product such as a plant, flower, fruit, vegetable, cereal, grain, pulse, mushroom spawn, pasture or lawn. The composition can be used to treat, for example, head blight and crown rot (*Fusarium*), facial eczema (*Pithomyces chartarum*), botrytis (*Botrytis cinerea*), leaf spot (*Septoria*, *Alteria*,
20 *Bipolaris*), powdery mildew (*Sphaerotheca macularis*, *Erysiphe*, *Sphaerotheca pannosa*), brown rot (*Monilinia fruticola*) and leaf/stem rust (*Puccinia*).

In a third embodiment, the subject is soil, timber, building material or a building. The composition can be used, for instance, in or on buildings for the treatment of mould, and on building materials and power poles for the treatment of fungal wood rot.

25 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.

30 According to a tenth aspect of the present invention, there is provided an antifungal composition comprising a synergistic combination of physiologically stressed *Arthrospira* and at least one fungi-static agent.

According to an eleventh aspect of the present invention, there is provided a method for preventing or treating a fungal infection or infestation of a subject, said

method comprising the step of administering to the subject an effective amount of a composition comprising a synergistic combination of physiologically stressed *Arthrospira* and at least one fungi-static agent.

5 Preferably, the method comprises the step of identifying a subject in need of such treatment or requiring such prevention.

There is also provided the use of physiologically stressed *Arthrospira* in combination with at least one fungi-static agent in the preparation of a medicament for the prevention or treatment of a fungal infection or infestation of a subject.

10 Any suitable fungi-static agent (monographed active) can be used, such as terbinafine, bifonazole, clotrimazole, miconazole, econazole, ketoconazole or tolnaftate. Any suitable concentration of monographed active can be used and will depend on the intended use for the composition.

Use of Physiologically Stressed *Arthrospira* as an Antibacterial

15 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-acetylmuramic acid) and peptidoglycan may be digestible by one or more of the bioactives.

20 According to a twelfth aspect of the present invention, there is provided a composition for use as an antibacterial, said composition comprising an effective amount of physiologically stressed *Arthrospira*.

According to a thirteenth aspect of the present invention, there is provided a method for preventing or treating a bacterial infection or infestation of a subject, 25 said method comprising the step of administering to the subject an effective amount of a composition comprising physiologically stressed *Arthrospira*.

Preferably, the method comprises the step of identifying a subject in need of such treatment or requiring such prevention.

30 There is also provided the use of physiologically stressed *Arthrospira* in the preparation of a medicament for the prevention or treatment of a bacterial infection or infestation of a subject.

Preferably, the bacteria is Gram-positive bacteria and may be, for example, *Bacilli*, *Clostridia*, *Staphylococci* or *Pneumococci*. The bacteria is preferably *Propionibacterium acne*, the causative agent of acne.

In a first embodiment, the subject is a human or an animal such as a mammal or vertebrate. In human subjects, the composition can be used to treat acne, dermatitis, ulcers or wounds caused by or infected with Gram-positive bacteria. The composition can be used to treat internal infections, such as those in the airways, in the mouth, in the digestive and in
5 genitourinary tracts (e.g. strep throat, middle ear infections).

In a second embodiment, the subject is an agricultural or horticultural product such as a plant, flower, fruit, vegetable, cereal, grain, pulse or mushroom spawn.

In a third embodiment, the subject is, for example, soil, a man-made structure, or a waterway in which bacteria can grow.

10 Use of Physiologically Stressed *Arthrospira* as a Pesticide

According to a fourteenth aspect of the present invention, there is provided a composition for use as a pesticide, said composition comprising an effective amount of physiologically stressed *Arthrospira*.

According to a fifteenth aspect of the present invention, there is provided a
15 method for suppressing or removing a pest, said method comprising administering or applying an effective amount of a composition comprising physiologically stressed *Arthrospira*.

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,
20 mosquitoes and termites. The pesticide can be used to stop the transmission of parasites, such as those causing malaria, and other insects-borne diseases.

In a first embodiment, the composition can be formulated as an insect repellent or bait.

In a second embodiment, the composition can be applied to an agricultural or
25 horticultural product such as a plant, flower, fruit, vegetable, cereal, grain, pulse or mushroom spawn.

In a third embodiment, the composition can be applied to, for example, soil, a man-made structure, a building, building material, a stream, a lake or wetlands.

Use of *Arthrospira* as a Therapeutic

30 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.

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.

5 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.

10 According to a sixteenth aspect of the present invention, there is provided a composition for repairing or preventing a defect of the skin of a mammal, said composition comprising an effective amount of *Arthrospira*.

Preferably, the composition comprises physiologically stressed *Arthrospira*.

According to a seventeenth aspect of the present invention, there is provided a method of repairing or preventing a defect of the skin of a mammal, said method comprising the application of an effective amount of a composition comprising *Arthrospira*.

15 Preferably, the composition comprises physiologically stressed *Arthrospira*.

Preferably, the method comprises the step of identifying a mammal in need of such treatment or requiring such prevention.

20 There is also provided the use of *Arthrospira*, preferably physiologically stressed *Arthrospira*, in the preparation of a medicament for repairing or preventing a defect of the skin of a mammal.

The compositions according to the sixteenth and seventeenth aspects of the invention can have one or more ingredients or properties as described for the compositions according to the other aspects of the invention that have been defined.

25 The compositions according to the sixteenth and seventeenth aspects of the invention are preferably prepared by any of the methods described above.

30 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.

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

BRIEF DESCRIPTION OF THE FIGURES

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*;

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

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

10 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*;

15 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;

20 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;

25 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;

30 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;

5 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;

10 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;

15 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;

20 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

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

BEST MODE AND OTHER MODES OF CARRYING OUT THE INVENTION

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*

30 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*

5 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

Standard amounts of AMYCOT® premix powder and the unprocessed powder were
10 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
15 be enzymic and could be due to one or more types of denaturable proteins.

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.

20 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.

25 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
30 in Figure 30. The optimum pH for activity was determined to be 6.

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

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.

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
60	12	10	11

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

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

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*

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

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Live cultures of target pathogens were sourced from the American Type Culture

- 5 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
<i>Trichophyton mentagrophytes</i>	4808, 9533	5068	
<i>Candida albicans</i>	753	4574	
<i>Trichophyton rubrum</i>			SN01 (Sullivan Niccolaides)
<i>Epidermophyton floccosum</i>			SN02 (Sullivan Niccolaides)
<i>Propionibacterium acne</i>	25746	5109	

- 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.

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

- 15 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).
- 20 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).

- 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*.

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	<i>Trichophyton mentagrophytes</i>	Clearance	6	10
4574		Clearance	6.5	-
5109	<i>Propionibacterium acne</i> (cultured anaerobically)	No clearance	-	-
ATCC No.				
4808	<i>Trichophyton mentagrophytes</i>	Clearance	5	8
9533	<i>Trichophyton mentagrophytes</i>	Clearance	6	10
753	<i>Candida albicans</i>	Clearance	6.5	-
25746	<i>Propionibacterium acne</i> (cultured anaerobically)	No clearance	-	-
Isolate No.				
SN01	<i>Trichophyton rubrum</i>	Clearance	6	-
SN02	<i>Epidermophyton floccosum</i>	Clearance	5	8
301	<i>Tinea pedis</i> - isolate	Clearance	6	10
307	Jockey itch-isolate	Clearance	6	10

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).

In the case of the anaerobic acne-causing bacteria *Propionibacterium acne*, 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 acne*

Respirometry showed that AMYCOT® has antibacterial activity against *P. acne*.

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. acne* (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. acne* 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.

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.

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

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.

Although *P. acne* 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

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.

Arthrospira (Spirulina) powders were obtained from the following suppliers:

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

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.

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).

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).

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

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*

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.

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

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.

5 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.

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.

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.

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.

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.

15 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.

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

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

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:

- 25 • *Fusarium graminearum*
- *Pithomyces Chartarum*
- *Botrytis cinerea*
- *Alternaria sp.*

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 was taken from the perimeter of the well to the outside perimeter of the zone.

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

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.

5 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.

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.

10 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

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
15 was only sparingly soluble.

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
20 inhibiting further sporulation.

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

The antifungal and antibacterial activities of various over-the-counter fungal and antibacterial creams (including Lamisil™, Dakta Gold™, Canesten™, Tinaderm™, 25 Daktarin™, Tripod Labs™, Resolve Tinea™, Resolve Balm™, Resolve Plus™, Clearasil™, Benzac W™) and AMYCOT® were tested in this and other examples.

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*
30 *floccosum*, *Trichophyton rubrum* and *Propionibacterium acne*.

The results are shown in Table 6.

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
<i>Trichophyton mentagrophytes</i>	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
<i>Epidermophyton floccosum</i>	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
<i>Trichophyton rubrum</i>	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
	4) Lamisil	Nil detected	1 mm clear, irregular	1.5 mm clear, irregular
<i>Propionibacterium acnes</i>	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®

5 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.

10 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.

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.

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.

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

Table 7 - Inhibitory Test (Figure 12)		
Pathogen: <i>Trichophyton rubrum</i>		
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: <i>Trichophyton mentagrophytes</i>		
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: <i>Candida albicans</i>		
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: <i>Candida albicans</i>		
Agent		Size of Clearance (mm)
Tripod Labs (clotrimazole 1% + tea tree oil)		10

Table 11 - Inhibitory Test (Figure 16)		
Pathogen: <i>Trichophyton mentagrophytes</i>		
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

5

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
6:00	10% tolnaftate	0
9:00	2% miconazole	9

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

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.

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.

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.

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

Table 17 - Fungicidal Test (Figure 22)		
Pathogen: <i>Trichophyton mentagrophytes</i>		
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: <i>Trichophyton rubrum</i>		
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: <i>Trichophyton mentagrophytes</i>		
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: <i>Trichophyton mentagrophytes</i>		
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: <i>Trichophyton mentagrophytes</i>		
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: <i>Candida albicans</i>		
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: <i>Trichophyton rubrum</i>		
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

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

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.

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.

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).

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®.

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).

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

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.

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.

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.

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

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.

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).

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

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

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

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

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

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®”.

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®”.

5 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.

10 It will be further appreciated that many changes can be made to the compositions, methods of use and preparation exemplified above without departing from the broad ambit and scope of the invention.

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.

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

CLAIMS

1. A composition for digesting or modifying chitin, a chitin derivative or a polymer having N-acetyl-D-glucosamine as a polymer subunit, said composition comprising physiologically stressed *Arthrospira*.

2. A method for digesting or modifying chitin, a chitin derivative or a polymer having N-acetyl-D-glucosamine as a polymer subunit, said method comprising the step of contacting chitin, chitin derivative or a polymer having N-acetyl-D-glucosamine as a polymer subunit with a composition comprising physiologically stressed *Arthrospira*.

3. A method for identifying a bioactive agent from *Arthrospira*, said method comprising the steps of:

(I) combining a composition comprising physiologically stressed *Arthrospira* with at least one test substrate modifiable by a bioactive agent from *Arthrospira*; and

(II) assaying for modification of the test substrate.

4. The method of claim 3, wherein the bioactive agent is 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.

5. A composition for use as a biocide and/or therapeutic, said composition comprising an effective amount of physiologically stressed *Arthrospira*.

6. A method for preventing or treating an infection or infestation of a subject by an organism, said method comprising the step of administering to the subject an effective amount of a composition comprising physiologically stressed *Arthrospira*.

7. The method of claim 6 further comprising the step of initially identifying a subject in need of such treatment or requiring such prevention.

8. A method for preparing an *Arthrospira*-based composition for use as a biocide and/or therapeutic, said method comprising the steps of:

(1) physiologically stressing *Arthrospira*; and

(2) combining the stressed *Arthrospira* of (1) with a suitable carrier, solvent, base or excipient.

9. The method of claim 8, wherein the method excludes the steps of explosively decompressing the physiologically stressed *Arthrospira* and/or freeze drying the physiologically stressing *Arthrospira* prior to adding the carrier, solvent, base or excipient.

10. An *Arthrospira*-based composition when prepared by the method of claim 9.

11. A method for preparing an *Arthrospira*-based composition for use as a biocide and/or therapeutic, said method comprising the steps:

(1) physiologically stressing *Arthrospira* by removing up to about 80% of liquid within which the *Arthrospira* is being grown;

5 (2) washing the stressed *Arthrospira* in order to remove contaminants;

(3) drying the washed *Arthrospira*; and

(4) combining the washed *Arthrospira* with a suitable carrier, solvent, base or excipient.

12. The method of claim 11, wherein the method excludes the steps of explosively decompressing the physiologically stressed *Arthrospira* and/or freeze drying the
10 physiologically stressing *Arthrospira* prior to adding the carrier, solvent, base or excipient.

13. An *Arthrospira*-based composition when prepared by the method of claim 12.

14. A composition for use as an antifungal, said composition comprising an effective amount of physiologically stressed *Arthrospira*.

15. A method for preventing or treating a fungal infection or infestation of
15 a subject, said method comprising the step of administering to the subject an effective amount of a composition comprising physiologically stressed *Arthrospira*.

16. The method of claim 15 further comprising the step of initially identifying a subject in need of such treatment or requiring such prevention.

17. The method of claim 16, when the subject is selected from the group
20 consisting of a human, an animal, an agricultural or horticultural product, soil, a man-made structure, and a waterway.

18. An antifungal composition comprising a synergistic combination of physiologically stressed *Arthrospira* and at least one fungi-static agent.

19. The antifungal composition of claim 18, wherein the fungi-static agent is
25 selected from the group consisting of terbinafine, bifonazole, clotrimazole, miconazole, econazole, ketoconazole and tolnaftate.

20. A method for preventing or treating a fungal infection or infestation of a subject, said method comprising the step of administering to the subject an effective amount of a composition comprising a synergistic combination of
30 physiologically stressed *Arthrospira* and at least one fungi-static agent.

21. The method of claim 20 further comprising the step of initially identifying a subject in need of such treatment or requiring such prevention.

22. The method of claim 20, wherein the fungi-static agent is selected from the group consisting of terbinafine, bifonazole, clotrimazole, miconazole, econazole, ketoconazole and tolnaftate.

23. A method for preparing the composition of claim 18, said method comprising
5 the steps of:

(1) physiologically stressing *Arthrospira*; and

(2) combining the stressed *Arthrospira* of (1) with the at least one fungi-static agent and a suitable carrier, solvent, base or excipient.

24. The method of claim 23, wherein the method excludes the steps of
10 explosively decompressing the physiologically stressed *Arthrospira* and/or freeze drying the physiologically stressing *Arthrospira* prior to adding the carrier, solvent, base or excipient.

25. A composition for use as an antibacterial, said composition comprising an effective amount of physiologically stressed *Arthrospira*.

26. A method for preventing or treating a bacterial infection or infestation
15 of a subject, said method comprising the step of administering to the subject an effective amount of a composition comprising physiologically stressed *Arthrospira*.

27. The method of claim 26 further comprising the step of initially identifying a subject in need of such treatment or requiring such prevention.

28. The method of claim 27, wherein the bacteria is *Propionibacterium acne*.

29. The method of claim 27, wherein the subject is selected from the group
20 consisting of a human, an animal, an agricultural or horticultural product, soil, a man-made structure, and a waterway.

30. A method for preparing the composition of claim 25, said method comprising the steps of:

25 (1) physiologically stressing *Arthrospira*; and

(2) combining the stressed *Arthrospira* of (1) with a suitable carrier, solvent, base or excipient.

31. The method of claim 30, wherein the method excludes the steps of
30 explosively decompressing the physiologically stressed *Arthrospira* and/or freeze drying the physiologically stressing *Arthrospira* prior to adding the carrier, solvent, base or excipient.

32. A composition for use as a pesticide, said composition comprising an effective amount of physiologically stressed *Arthrospira*.

33. A method for suppressing or removing a pest, said method comprising administering or applying an effective amount of a composition comprising physiologically stressed *Arthrospira*.

34. The method of claim 33, wherein the pest is selected from the group
5 consisting of a crustacean, acari, insect and parasite.

35. A method for preparing the composition of claim 32, said method comprising the steps of:

(1) physiologically stressing *Arthrospira*; and

(2) combining the stressed *Arthrospira* of (1) with a suitable carrier, solvent, base or
10 excipient.

36. The method of claim 35, wherein the method excludes the steps of explosively decompressing the physiologically stressed *Arthrospira* and/or freeze drying the physiologically stressing *Arthrospira* prior to adding the carrier, solvent, base or excipient.

37. A composition for repairing or preventing a defect of the skin of a
15 mammal, said composition comprising an effective amount of *Arthrospira*.

38. The composition of claim 37, wherein the composition comprises physiologically stressed *Arthrospira*.

39. A method of repairing or preventing a defect of the skin of a mammal, said method comprising the application of an effective amount of a composition comprising
20 *Arthrospira*.

40. The method of claim 39, wherein the composition comprises physiologically stressed *Arthrospira*.

41. The method of claim 39 further comprising the step of initially identifying a mammal in need of such treatment or requiring such prevention.

25 42. A method for preparing the composition of claim 38, said method comprising the steps of:

(1) physiologically stressing *Arthrospira*; and

(2) combining the stressed *Arthrospira* of (1) with a suitable carrier, solvent, base or
excipient.

30 43. The method of claim 42, wherein the method excludes the steps of explosively decompressing the physiologically stressed *Arthrospira* and/or freeze drying the physiologically stressing *Arthrospira* prior to adding the carrier, solvent, base or excipient.

44. The method of claim 40, wherein the skin defect is selected from the group consisting of 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, and cracked heels and elbows.

45. The method of claim 9, wherein the *Arthrospira* is *A. maxima*.