

COMPOSITIONS CONTAINING A HOLLOW GLUCAN PARTICLE OR A CELL WALL PARTICLE
ENCAPSULATING A TERPENE COMPONENT, METHODS OF MAKING AND USING THEM

4 The present invention relates to compositions
5 comprising terpenes and hollow glucan particles or
6 cell wall particles and methods for preparing such
7 compositions. The compositions increase terpene
8 stability and activity and provide a suitable carrier
9 for the terpenes. The invention also relates to
10 methods of using such compositions in the medical,
11 veterinary and agricultural fields.

12

13 Terpenes are chemical compounds that are widespread
14 in nature, mainly in plants as constituents of
15 essential oils. Their building block is the
16 hydrocarbon isoprene $(C_5H_8)_n$. Examples of terpenes
17 include citral, pinene, nerol, b-ionone, geraniol,
18 carvacrol, eugenol, carvone, terpeniol, anethole,
19 camphor, menthol, limonene, nerolidol, farnesol,
20 phytol, carotene (vitamin A_1), squalene, thymol,

1 tocotrienol, perillyl alcohol, borneol, myrcene,
2 simene, carene, terpenene, and linalool.

3

4 Terpenes are classified as Generally Recognized as
5 Safe (GRAS) and have been used for many years in the
6 flavouring and aroma industries. The LD₅₀ in rats of
7 citral is approximately 5 g/kg, which is a further
8 indication of the relative safety of these compounds.
9 Furthermore, terpenes have a relatively short life
10 span of approximately 28 days once exposed to oxygen
11 (e.g. air). Terpenes will decompose to CO₂ and
12 water. This decomposition or break down of terpenes
13 demonstrates the safety and environmental
14 friendliness of the compositions and methods of the
15 invention.

16

17 Terpenes have been found to inhibit the growth of
18 cancerous cells, decrease tumour size, decrease
19 cholesterol levels, and have a biocidal effect on
20 micro-organisms *in vitro*. Owawunmi, (Letters in
21 Applied Microbiology, 1993, 9(3): 105-108), showed
22 that growth media with more than 0.01% citral reduced
23 the concentration of *E. coli*, and at 0.08% there was
24 a bactericidal effect. U.S. Patent No. 5,673,468
25 describes a terpene formulation, based on pine oil,
26 used as a disinfectant or antiseptic cleaner. U.S.
27 Patent No. 5,849,956 teaches that a terpene found in
28 rice has antifungal activity. U.S. Patent No.
29 5,939,050 describes an oral hygiene antimicrobial
30 product with a combination of 2 or 3 terpenes that
31 showed a synergistic effect. Several U.S. Patents
32 (U.S. Patent Nos. 5,547,677, 5,549,901, 5,618,840,

1 5,629,021, 5,662,957, 5,700,679, 5,730,989) teach
2 that certain types of oil-in-water emulsions have
3 antimicrobial, adjuvant, and delivery properties.
4 Terpenes have been found to be effective and nontoxic
5 dietary anti-tumor agents, which act through a
6 variety of mechanisms of action (Crowell et al.
7 Crit. Rev. Oncog., 1994, 5(1): 1-22; Crowell et al.
8 Adv. Exp. Med. Biol., 1996, 401: 131-136). The
9 terpenes geraniol, tocotrienol, perillyl alcohol, b-
10 ionone, and d-limonene, suppress hepatic HMG-CoA
11 reductase activity, a rate limiting step in
12 cholesterol synthesis, and modestly lower cholesterol
13 levels in animals (Elson et al, J. Nutr., 1994, 124:
14 607-614). D-limonene and geraniol reduced mammary
15 tumors (Elegbede et al. Carcinogenesis, 1984, 5(5):
16 661-664; Elegbede et al., J. Natl. Cancer Inst.,
17 1986, 76(2): 323-325; Karlson et al. Anticancer
18 Drugs, 1996, 7(4): 422-429) and suppressed the growth
19 of transplanted tumors (Yu et al., J. Agri. Food
20 Chem., 1995, 43: 2144-2147).

21
22 Terpenes have also been found to inhibit the *in vitro*
23 growth of bacteria and fungi (Chaumont et al.), Ann.
24 Pharm. Fr., 1992, 50(3): 156-166; Moleyar et al.,
25 Int. J. Food Microbiol, 1992, 16(4): 337-342; and
26 Pattnaik et al. Microbios, 1997, 89(358): 39-46) and
27 some internal and external parasites (Hooser et al.,
28 J. Am. Vet. Med. Assoc., 1986, 189(8): 905-908).
29 Geraniol was found to inhibit growth of *Candida*
30 *albicans* and *Saccharomyces cerevisiae* strains by
31 enhancing the rate of potassium leakage and
32 disrupting membrane fluidity (Bard et al., Lipids,

1 1998, 23(6): 534-538). B-ionone has antifungal
2 activity which was determined by inhibition of spore
3 germination, and growth inhibition in agar (Mikhlin
4 et al., A. Prikl. Biokhim. Mikrobiol, 1983, 19: 795-
5 803; Salt et al., Adam. Physiol. Molec. Plant Path,
6 1986, 28: 287-297). Teprenone
7 (geranylgeranylacetone) has an antibacterial effect
8 on *H. pylori* (Ishii, Int. J. Med. Microbiol. Virol.
9 Parasitol. Infect. Dis., 1993, 280(1-2): 239-243).
10 Rosanol, a commercial product with 1% rose oil, has
11 been shown to inhibit the growth of several bacteria
12 (*Pseudomonas*, *Staphylococcus*, *E. coli*, and *H. pylori*).
13 Geraniol is the active component (75%) of rose oil.
14 Rose oil and geraniol at a concentration of 2 mg/L
15 inhibited the growth of *H. pylori* *in vitro*. Some
16 extracts from herbal medicines have been shown to
17 have an inhibitory effect in *H. pylori*, the most
18 effective being decursinol angelate, decursinol,
19 magnolol, berberine, cinnamic acid, decursinol, and
20 gallic acid (Bae et al., Biol. Pharm. Bull., 1998,
21 21(9) 990-992). Extracts from cashew apple,
22 anacardic acid, and (E)-2-hexenal have shown
23 bactericidal effect against *H. pylori*.
24
25 Diterpenes, i.e., trichorabdol A (from *R.*
26 *Trichocarpa*), have shown a very strong antibacterial
27 effect against *H. pylori* (Kadota et al., Zentralbl.
28 Bakteriologie, 1997, 287(1): 63-67).
29
30 Solutions of 11 different terpenes were effective in
31 inhibiting the growth of pathogenic bacteria in *in*
32 *vitro* tests; levels ranging between 100 ppm and 1000

1 ppm were effective. The terpenes were diluted in
2 water with 1% polysorbate 20 (Kim et al., J. Agric.
3 Food Chem., 1995, 43: 2839-2845).

4
5 There may be different modes of action of terpenes
6 against microorganisms; they could (1) interfere with
7 the phospholipid bilayer of the cell membrane, (2)
8 impair a variety of enzyme systems (HMG-reductase),
9 and (3) destroy or inactivate genetic material. It
10 is believed that due to the modes of action of
11 terpenes being so basic, e.g., blocking of
12 cholesterol, that infective agents will not be able
13 to build a resistance to terpenes.

14
15 There are, however, a number of drawbacks to the use
16 of terpenes. These include:

- 17 - Terpenes are liquids which can make them difficult
18 to handle and unsuitable for certain purposes.
- 19 - Terpenes are not very miscible with water, and it
20 generally requires the use of detergents,
21 surfactants or other emulsifiers to prepare
22 aqueous emulsions. A stable solution can,
23 however, be obtained by mixing the terpenes under
24 high shear.
- 25 - Dry powder terpene formulations generally only
26 contain a low percentage w/w of terpenes.
- 27 - Terpenes are prone to oxidation in aqueous
28 emulsion systems, which make long term storage a
29 problem.

30
31 There are limitations to the current techniques of
32 spray coating, extrusion, coacervation, molecular

1 encapsulation, and spray drying/cooling to provide
2 ingredient delivery systems.

3

4 Baker's yeast cell walls are derived from baker's
5 yeast cells and are composed of the insoluble
6 biopolymers β -1,3-glucan, β -1,6-glucan, mannan and
7 chitin. They are typically 2-4 micron in diameter
8 microspheres with a shell wall that is only 0.2-0.3
9 micron thick surrounding an open cavity. This
10 material has considerable liquid holding capacity,
11 typically absorbing 5-25 times its weight in liquid.
12 The shell is sufficiently porous that payloads up to
13 150,000 Daltons in size can pass through the outer
14 shell and be absorbed into the hollow cavity of the
15 spherical particle. Baker's yeast cell walls have
16 several unique properties, including heat stability
17 (e.g. to 121°C), shear stability, pH stability (e.g.
18 pH 2-12), and at high concentrations they do not
19 build significant viscosity. In addition to its
20 physical properties this composition contains natural
21 and healthy dietary fibres that deliver
22 cardiovascular and immunopotential health
23 benefits.

24

25 Yeast cell walls are prepared from yeast cells by the
26 extraction and purification of the insoluble
27 particulate fraction from the soluble components of
28 the yeast cell. The fungal cell walls can be
29 produced from the insoluble byproduct of yeast
30 extract manufacture. Further, the yeast cells can be
31 treated with an aqueous hydroxide solution, without
32 disrupting the yeast cell walls, which digests the

1 protein and intracellular portion of the cell,
2 leaving the yeast cell wall component devoid of
3 significant protein contamination, and having
4 substantially the unaltered cell wall structure of
5 $\beta(1-6)$ and $\beta(1-3)$ linked glucans. A more detailed
6 description of whole glucan particles and the process
7 of preparing them is described by Jamas et al. in
8 U.S. Pat. No. 4,810,646 and in co-pending patent
9 applications U.S. Ser. No. 166,929, U.S. Ser. No.
10 297,752 and U.S. Ser. No. 297,982. US Patent No.
11 6,242,594, assigned to Novogen Research Pty Ltd.,
12 describes a method of preparing yeast glucan
13 particles by alkali extraction, acid extraction and
14 then extraction with an organic solvent and finally
15 drying. US 5,401,727, assigned to AS Biotech-
16 Mackzymal, discloses the methods of obtaining yeast
17 glucan particles and methods of using them to promote
18 resistance in aquatic animals and as an adjuvant for
19 vaccinations. US 5,607,677, assigned to Alpha-Beta
20 Technology Inc., discloses the use of hollow whole
21 glucan particles as a delivery package and adjuvant
22 for the delivery of a variety of pharmaceutical
23 agents. The teachings of the abovementioned patents
24 and applications are incorporated herein by
25 reference.

26

27 Other types of yeast and fungi cells have cell walls
28 that do not contain glucan. The cell walls of such
29 yeast and fungi can be isolated by similar techniques
30 to those mentioned above to obtain cell wall
31 particles.

32

1 Additionally, the cells of many plants, algae,
2 bacteria and other micro-organisms also comprise a
3 cell wall. The structure and composition of the cell
4 wall varies between micro-organism, but in general it
5 is a robust and relatively inert structure. It is
6 possible to obtain cell wall particles derived from
7 such cells through conventional techniques, such as
8 those mentioned above in relation to yeast.

9

10 We have now found that terpenes can be taken up and
11 stably encapsulated within hollow glucan particles or
12 cell wall particles. Encapsulation of terpenes into
13 such particles can be achieved by incubation of the
14 particles with the terpene.

15

16 According to the present invention there is provided
17 a composition comprising a hollow glucan particle or
18 a cell wall particle encapsulating a terpene
19 component.

20

21 The term "hollow glucan particle" as used herein
22 includes any hollow particle comprising glucan as a
23 structural component. Thus, in particular, the term
24 includes yeast cell walls (in purified or crude
25 forms) or hollow whole glucan particles. The term
26 "cell wall particle" refers to a particle comprising
27 the wall of a cell (in a purified or crude form),
28 wherein glucan is not a structural component.
29 Suitable particles include the cell walls of plant,
30 algal, fungal or bacterial cells. Cell wall
31 particles generally retain the shape of the cell from
32 which they are derived, and thus, like a hollow

1 glucan particle, provide a hollow central cavity
2 suitable for encapsulating the terpene component.

3

4 For the present invention it is necessary that the
5 hollow glucan particle or cell wall particle is able
6 to stably encapsulate the terpene component. In
7 general this means the hollow glucan particle or cell
8 wall particle must be able to maintain its structure
9 during incubation with the terpene component
10 (generally the terpene component is at a relatively
11 high concentration), and that terpene component must
12 be able to migrate into the particle. Hollow glucan
13 particles and cell wall particles are generally
14 formed from relatively inert materials and are
15 porous, and thus it can be assumed that, in general,
16 hollow glucan particles and cell wall particles will
17 be able to encapsulate a terpene component.

18

19 Compositions according to the present invention are
20 effective against various infective agents including
21 bacteria, viruses, mycoplasmas, fungi and/or
22 nematodes.

23

24 The compositions according to the present invention
25 can provide the following advantages:

- 26 - maximise terpene payload;
- 27 - minimise unencapsulated payload;
- 28 - control payload stability;
- 29 - control payload release kinetics;
- 30 - creation of a solid form of a liquid terpene to
- 31 increase the mass and uniformity;
- 32 - simplify handling and application of terpenes; and

1 - mask the smell and taste of the terpene.

2

3 Particularly suitable hollow glucan particles or cell
4 wall particles are fungal cell walls, preferably
5 yeast cell walls. Yeast cell walls are preparations
6 of yeast cells that retain the three-dimensional
7 structure of the yeast cell from which they are
8 derived. Thus they have a hollow structure which
9 allows the terpene component to be encapsulated
10 within the yeast cell walls. The yeast walls may
11 suitably be derived from Baker's yeast cells
12 (available from Sigma Chemical Corp., St. Louis,
13 MO). Yeast cell wall particles with desirable
14 properties can also be obtained from Biorigin (Sao
15 Paolo, Brazil) under the trade name Nutricell MOS 55.
16 These particles are a spray dried extract of *S.*
17 *cerevisiae*.

18

19 Alternative particles are those known by the trade
20 names SAF-Mannan (SAF Agri, Minneapolis, MN) and
21 Nutrex (Sensient Technologies, Milwaukee, WI). These
22 are hollow glucan particles that are the insoluble
23 waste stream from the yeast extract manufacturing
24 process. During the production of yeast extracts the
25 soluble components of partially autolyzed yeast cells
26 are removed and the insoluble residue is a suitable
27 material for terpene loading. These hollow glucan
28 particles comprise approximately 25-35% beta
29 1,3-glucan w/w. A key attribute of these materials
30 are that they contain more than 10% lipid w/w and are
31 very effective at absorbing terpenes. In addition,

1 as a waste stream product they are a relatively cheap
2 source of hollow glucan particles.

3
4 Alternative hollow glucan particles which have higher
5 purity are those produced by Nutricepts (Nutricepts
6 Inc., Burnsville, MN) and ASA Biotech. These
7 particles have been alkali extracted, which removes
8 additional intracellular components as well as
9 removes the outer mannoprotein layer of the cell wall
10 yielding a particle of 50-65% glucan w/w.

11
12 Higher purity hollow glucan particles are the WGP
13 particles from Biopolymer Engineering. These
14 particles are acid extracted removing additional
15 yeast components yielding a product 75-85% glucan
16 w/w.

17
18 Very high purity hollow glucan particles are Adjuvax™
19 from Alpha-beta Technology, Inc. (Worcester, MA) and
20 microparticulate glucan from Novogen (Stamford, CT).
21 These particles are organic solvent extracted which
22 removes residual lipids and so the particles comprise
23 more than 90% glucan w/w.

24
25 In some embodiments a high purity glucan particle or
26 cell wall particle may be required, for example where
27 strict control over possible contaminants is
28 required. In these instances the higher purity
29 particles would be preferred over other less pure
30 products. For other embodiments, the less pure
31 particles would be preferred for economic reasons;

1 those particles have also been found to be more
2 effective at absorbing terpenes.

3
4 Preferably the hollow glucan particle or cell wall
5 particle has a slight lipid content, such as 1 or 2%
6 w/w lipid. A slight lipid content can increase the
7 ability of the particle to encapsulate the terpene
8 component. Preferably the lipid content of the
9 hollow glucan particle or cell wall particle is 5%
10 w/w or greater, more preferably 10% w/w or greater.

11
12 Optionally the terpene component of the present
13 invention can be associated with a surfactant. The
14 surfactant can be non-ionic, cationic, or anionic.
15 Examples of suitable surfactants include sodium
16 lauryl sulphate, polysorbate 20, polysorbate 80,
17 polysorbate 40, polysorbate 60, polyglyceryl ester,
18 polyglyceryl monooleate, decaglyceryl monocaprylate,
19 propylene glycol dicaprilate, triglycerol
20 monostearate, polyoxyethylenesorbitan, monooleate,
21 Tween®, Span® 20, Span® 40, Span® 60, Span® 80, Brig
22 30 or mixtures thereof. The surfactant acts to hold
23 the terpene component in an emulsion and also assists
24 encapsulation of the terpene component into the
25 hollow glucan particle or cell wall particle.

26
27 The terpene component of the present invention can
28 comprise a single terpene or a mixture of terpenes.
29 Mixtures of terpenes can result in synergistic
30 effects.

31

1 The term "terpene" as used herein refers not only to
2 terpenes of formula $(C_5H_8)_n$, but also encompasses
3 terpene derivatives, such as terpene aldehydes or
4 terpene polymers. Natural and synthetic terpenes are
5 included, for example monoterpenes, sesquiterpenes,
6 diterpenes, triterpenes, and tetraterpenes. In
7 addition, reference to a single name of a compound
8 will encompass the various isomers of that compound.
9 For example, the term citral includes the cis-isomer
10 citral-a (or geranial) and the trans-isomer citral-b
11 (or neral).

12
13 It should be noted that terpenes are also known by
14 the names of the extract or essential oil which
15 contain them, e.g. lemongrass oil (contains citral).

16
17 The terpenes which are exempted from US regulations
18 and which are listed in EPA regulation 40 C.F.R. Part
19 152 (incorporated herein by reference in its
20 entirety) are suitable for use in this invention.

21
22 Particularly suitable terpenes for use in the present
23 invention include those selected from the group
24 consisting of citral, pinene, nerol, b-ionone,
25 geraniol, carvacrol, eugenol, carvone (for example L-
26 carvone), terpeniol, anethole, camphor, menthol,
27 thymol, limonene, nerolidol, farnesol, phytol,
28 carotene (vitamin A₁), squalene, thymol, tocotrienol,
29 perillyl alcohol, borneol, myrcene, simene, carene,
30 terpenene, linalool and mixtures thereof.

31

1 Preferably the terpenes used in the present invention
2 have the general structure $C_{10}H_{16}$ as this sub-group is
3 generally more effective against infective agents.

4

5 More preferably the terpene component comprises a
6 terpene selected from the group consisting of
7 geraniol, thymol, citral, carvone (for example L-
8 carvone), eugenol and b-ionone.

9

10 The terpene component can suitably comprise thymol,
11 as this terpene has been shown to be particularly
12 effective in treating or preventing fungal plant
13 infections.

14

15 Another particularly suitable terpene is citral which
16 has demonstrated particular efficacy against a number
17 of micro-organisms.

18

19 A combination of geraniol, thymol and eugenol has
20 demonstrated particular efficacy in combating plant
21 infections, and is thus a particularly suitable
22 terpene component.

23

24 Other terpene formulations which have shown high
25 efficacy in treating plant infections include
26 (percentages are w/w):

- 27 - 100% thymol;
- 28 - 50% geraniol and 50% thymol;
- 29 - 50% eugenol and 50% thymol;
- 30 - 33% geraniol, 33% eugenol and 33% thymol;
- 31 - 33% eugenol, 33% thymol and 33% citral;
- 32 - 25% geraniol, 25% eugenol, 25% thymol and

1 25% citral;
2 - 20% geraniol, 20% eugenol, 20% citral, 20%
3 thymol and 20% L-carvone.

4
5 Accordingly a terpene component comprising any of the
6 above formulations is particularly suitable for use
7 in the present invention.

8
9 In one embodiment the terpene component includes one
10 or more terpenes which contain oxygen. Citral, for
11 example citral 95, is an oxygenated $C_{10}H_{16}$ terpene,
12 $C_{10}H_{16}O$ CAS No. 5392-40-5 (3,7-dimethyl-2,6-octadien-
13 1-ol). A stable suspension of citral can be formed
14 up to about 2500 ppm. Citral can be made into a
15 solution at up to about 500 ppm. A stable suspension
16 of hollow glucan particles incorporating citral of 25
17 ppt citral can be made.

18
19 The composition of the invention can comprise 1 to
20 99% by volume terpenes, 0 to 99% by volume surfactant
21 and 1 to 99% hollow glucan particles or cell wall
22 particles. More specifically the composition can
23 comprise about 10% to about 67% w/w terpenes, about
24 0.1-10% surfactant and about 40-90% hollow glucan
25 particles or cell wall particles.

26
27 Suitably a composition of the present invention
28 comprises from about 500 to about 10,000 ppm hollow
29 glucan particles or cell wall particles, where the
30 particles contain from about 1 to about 67% terpene
31 component. Preferably the composition comprises from
32 about 1000 to about 2000 ppm hollow glucan particles

1 or cell wall particles, where the particles contain
2 from about 10 to about 50% terpene component.

3

4 Specific compositions can include e.g., for bacteria
5 and fungi, hollow glucan particles or cell wall
6 particles encapsulating terpenes in water or standard
7 0.9% saline with up to 67% L-carvone, up to 67%
8 eugenol, up to 67% citral, up to 67% thymol and L-
9 carvone, up to 67% geraniol, or up to 67% citral and
10 L-carvone and eugenol, and 1% Tween® 80; for mold,
11 hollow glucan particles or cell wall particles
12 encapsulating terpenes in water or standard 0.9%
13 saline with up to 67% citral and 1% Tween® 80; or for
14 mycoplasma, hollow glucan particles or cell wall
15 particles encapsulating terpenes in water or standard
16 0.9% saline with up to 67% citral, up to 67% L-
17 carvone and eugenol, up to 67% eugenol, up to 67 %
18 geraniol, or up to 67% geraniol, thymol, and 1%
19 Tween® 80.

20

21 Concentrations of hollow glucan particles or cell
22 wall particles encapsulating terpenes of 1, 5, 10,
23 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 125, 130,
24 140, 150, 160, 175, 190, 200, 225, 250, 275, 300,
25 325, 350, 375, 400, 425, 450, 475, 500, 525, 550,
26 575, 600, 625, 650, 675, 700, 725, 750, 775, 800,
27 825, 850, 875, 900, 925, 950, 975, 1000, 1100, 1250,
28 1375, 1425, 1500, 1600, 1750, or 2000 ppm can be used
29 as effective concentrations in the compositions and
30 methods of the current invention. Even higher
31 concentrations (up to 25 ppt, i.e. parts per

1 thousand) can be made and may be useful in the
2 current invention.

3

4 The composition of the present invention can comprise
5 between about 1 ppm and about 25 ppt (25000 ppm) of
6 the terpene component, preferably 100 to 2000 ppm of
7 the terpene component, for example, 250, 500, 1000,
8 2000 ppm thereof.

9

10 The terpenes, surfactants, and other components of
11 the invention may be readily purchased or synthesised
12 using techniques generally known to synthetic
13 chemists.

14

15 It is highly preferred that terpenes used in the
16 present invention, for safety and regulatory reasons,
17 are at least food grade terpenes (as defined by the
18 United States FDA or equivalent national regulatory
19 body outside the USA).

20

21 Optionally the composition can comprise other food-
22 grade active compounds in addition to the terpene
23 component, for example other antimicrobial agents,
24 enzymes, or the like.

25

26 Optionally the composition can comprise a further
27 active agents in addition to the terpene component,
28 for example an antimicrobial agent, an anti-fungal
29 agent, an insecticidal agent, an anti-inflammatory
30 agent, an anaesthetic or the like. Suitable agents
31 include:

- 1 - Anti-fungal: Cell wall hydrolyases (assuming they
- 2 do not degrade the hollow glucan particle or cell
- 3 wall particle), cell wall synthesis inhibitors,
- 4 standard antifungals.
- 5 - Anti-bacterial: Antiseptics, cell wall hydrolases,
- 6 synthesis inhibitors, antibiotics.
- 7 - Insecticidal: Natural insecticides, chitinase.

8

9 The composition can comprise an antioxidant to reduce

10 oxidation of the terpene. An example of such an

11 anti-oxidant might be rosemary oil, vitamin C or

12 vitamin E.

13

14 The composition of the present invention can be in

15 the form of a dry powder. The composition can be

16 provided in combination with an agriculturally, food

17 or pharmaceutically acceptable carrier or excipient

18 in a liquid, solid or gel-like form.

19

20 For solid compositions, suitable carriers include

21 pharmaceutical grades of mannitol, lactose, starch,

22 magnesium stearate, sodium saccharin, talc,

23 cellulose, glucose, sucrose, magnesium carbonate, and

24 the like. Suitably the formulation is in tablet or

25 pellet form. As suitable carrier could also be a

26 human or animal food material. Additionally,

27 conventional agricultural carriers could also be

28 used.

29

30 A pellet, tablet or other solid form of the

31 composition can preferably also contain a dispersal

32 agent which promotes dispersal of the composition

1 when placed into a liquid, e.g. water. Suitable
2 dispersal agents include xanthan gum, maltodextrin,
3 alginates, or the like.

4
5 Liquid compositions can, for example, be prepared by
6 dispersing the composition in water, saline, aqueous
7 dextrose, glycerol, ethanol, or the like, to form a
8 solution or suspension. If desired, these
9 compositions can contain minor amounts of non-toxic
10 auxiliary substances such as wetting or emulsifying
11 agents, pH buffering agents (for example, sodium
12 acetate, sorbitan monolaurate, triethanolamine sodium
13 acetate or triethanolamine oleate). The methods of
14 preparing such liquid compositions are known, or will
15 be apparent, to those skilled in this art; for
16 example see Remington: The Science and Practice of
17 Pharmacy; Lippincott, Williams & Wilkins; (December
18 15, 2000) - which is incorporated herein by
19 reference. Again a liquid composition could be
20 prepared by dispersing the composition in a liquid
21 human or animal food or drink material. Additionally
22 a suitable liquid agricultural excipient could be
23 used.

24
25 For oral administration tablets and granules are
26 generally preferred. Tablets may contain binders and
27 lubricants. Fine powders or granules may contain
28 diluting, dispersing and/or surface active agents and
29 can be presented in water or in a syrup. Capsules or
30 sachets can conveniently contain the composition in a
31 dry state. Non-aqueous solutions or suspensions of
32 the composition are also suitable and may contain

1 suspending agents. Where desirable or necessary,
2 flavouring, preserving, suspending, thickening, or
3 emulsifying agents can be included. Of course, it
4 would be suitable to use a food or drink material as
5 an oral delivery method.

6
7 Parental administration is generally characterised by
8 injection. For injectables it will be appreciated
9 that, in general, all materials used in the
10 composition and any excipient used must be of
11 pharmaceutical grade. Injectables can be prepared in
12 conventional forms, either as liquid solutions,
13 emulsions or suspensions, solid forms suitable for
14 dissolution, suspension in liquid prior to injection,
15 or as emulsions. An alternative approach for
16 parental administration involves use of a slow
17 release or sustained release system, such that a
18 constant level of dosage is maintained. See, for
19 example, U.S. Patent No. 3,710,795, which is
20 incorporated by reference herein. Preparations for
21 parenteral can also contain buffers, diluents and
22 other suitable additives. Examples of non-aqueous
23 solvents are propylene glycol, polyethylene glycol,
24 vegetable oils (such as olive oil), and injectable
25 organic esters (such as ethyl oleate). Aqueous
26 carriers include water, alcoholic/aqueous solutions,
27 emulsions, or suspensions, including saline and
28 buffered media. Other parenteral vehicles include
29 sodium chloride solution, Ringer's dextrose, dextrose
30 and sodium chloride, lactated Ringer's, or fixed
31 oils. Vehicles for intravenous use include fluid and
32 nutrient replenishers, electrolyte replenishers (such

1 as those based on Ringer's dextrose) and the like.
2 Preservatives and other additives can also be present
3 such as, for example, antimicrobials, anti-oxidants,
4 chelating agents, inert gases, and the like.

5
6 For topical administration liquids, suspension,
7 lotions, creams, gels, ointments, drops,
8 suppositories, sprays and powders may be used.
9 Conventional pharmaceutical carriers, aqueous, powder
10 or oily bases, thickeners, and the like can be used
11 as necessary or desirable.

12
13 The present invention further provides a method of
14 preparing a hollow glucan particle or cell wall
15 particle encapsulating a terpene component, said
16 method comprising the steps of;

- 17 a) providing a terpene component;
- 18 b) providing a hollow glucan particle or cell
19 wall particle;
- 20 c) incubating the terpene component with the
21 glucan particle or cell wall particle under
22 suitable conditions for terpene
23 encapsulation; and
- 24 d) recovering the hollow glucan particle or
25 cell wall particle encapsulating the terpene
26 component.

27
28 Optionally the above method can further comprise the
29 step of drying the particles encapsulating the
30 terpene component. Drying may be achieved in a
31 number of ways and mention may be made of freeze

1 drying, fluidised bed drying, drum drying or spray
2 drying, all of which are well known processes.

3

4 In step a) of the above method, the terpene component
5 is suitably provided as a suspension in an aqueous
6 solvent, and optionally in the presence of a
7 surfactant. Suitably the solvent is water. A
8 suitable surfactant is Tween-80
9 (polyoxyethylenesorbitan monooleate), and preferably
10 the surfactant is present at a concentration of about
11 0.1 to 10 % by volume of the total reaction mixture,
12 more preferably about 1%. Alternatively the terpene
13 component may be provided as a true solution in a
14 solvent, e.g. water. A true solution of terpene in
15 water can be obtained by mixing the terpene in water
16 at high shear until a true solution is obtained.
17 Publication No WO 03/020024 provides further details
18 of forming true solutions of terpenes in water.

19

20 In step b) of the above method, the hollow glucan
21 particle or cell wall particle is suitably provided
22 as a suspension in water or other suitable liquid.
23 Suitably the suspension comprises approximately 1 to
24 1000 mg particles per ml, preferably 200 to 400
25 mg/ml. Alternatively the particles may be provided
26 as a dry powder and added to the terpene-surfactant
27 suspension.

28

29 Alternatively the particles are provided in
30 sufficient liquid to minimally hydrate the particles,
31 but not in significant excess. The term
32 "hydrodynamic volume" (HV) is used to describe the

1 volume of liquid required to minimally hydrate the
2 particles. Thus suitably the particles are provided
3 with a volume ranging from the HV and a volume of 1.5
4 times the HV (1.5HV). This makes the subsequent
5 drying step more efficient. Also, where a low volume
6 of liquid is used (ie. around HV to 1.5HV), it is
7 also possible to extrude the finished product into
8 pellet or noodle form, which is convenient for
9 fluidised bed drying.

10

11 It has been found that the terpene component can
12 become encapsulated by the hollow glucan particle or
13 cell wall particle at room temperature. The rate of
14 encapsulation is, however, increased at 37°C but the
15 temperature should be kept below the boiling point or
16 denaturing temperature of any component of the
17 composition. Suitable conditions for step c) of the
18 above method are therefore atmospheric pressure at a
19 temperature of 20 to 37°C. Optimisation of the
20 conditions for a particular encapsulation reaction
21 will be a matter of routine experimentation.

22

23 The present invention further provides a method of
24 killing a microorganism, said method comprising the
25 step of;

26 - contacting said microorganism with a composition
27 comprising a hollow glucan particle or cell wall
28 particle encapsulating a terpene component.

29

30 Suitable compositions are those defined in more
31 detail above.

1 The present invention further provides a method of
2 preventing or treating an infection in a patient,
3 said method comprising the step of;
4 a) administering to said patient in a
5 therapeutically effective dose, a composition
6 comprising a hollow glucan particle or cell wall
7 particle encapsulating a terpene component.

8
9 Suitable compositions are those defined in more
10 detail above.

11
12 The infection of the patient may be caused by any
13 infectious agent. Examples of these infectious
14 agents include, but are not restricted to
15 *Staphylococcus aureus*, *Aspergillius fumigatus*,
16 *Mycoplasma iowae*, *Penicillium sp.*, and *Mycoplasma*
17 *pneumoniae*.

18
19 For internal administration the composition may be
20 administered orally, vaginally, rectally, by
21 inhalation, or by parenteral routes, e.g. by
22 intradermal, subcutaneous, intramuscular,
23 intraperitoneal, intrarectal, intraarterial,
24 intralymphatic, intravenous, intrathecal and
25 intratracheal routes. Suitable formulations of the
26 composition for these routes are discussed above.

27
28 For external treatment, the composition may be
29 applied topically, for example as a cream or ointment
30 or as a dry powder for treatment of a wound.

31

1 The amount of terpene administered in the above
2 method should clearly be sufficient to achieve the
3 desired result, i.e. prevention and/or treatment of
4 the infection, but should not be at a level which
5 will induce serious toxic effects in the patient.
6

7 The amount of composition administered will, of
8 course, be dependent on the manner of administration,
9 on the patient being treated, i.e. their weight,
10 their age, condition, sex and extent of the disease
11 in the subject and on the judgement of the
12 prescribing physician. The dose, schedule of doses,
13 and route of administration can be varied. One of
14 skill in the art would readily be able to determine
15 an anti-infective amount for a given application
16 based on the general knowledge in the art and the
17 procedures in the Examples given below. It should be
18 noted that the term "patient" as used herein refers
19 to any individual, either human or animal, to which
20 the treatment is applied. Thus, the patient can be a
21 domesticated animal (e.g., cat, dog, etc.), livestock
22 (e.g., cattle, horse, pig, sheep, goat, etc.),
23 laboratory animal (e.g., mouse, rabbit, rat, guinea
24 pig, etc.), birds and fish. Suitably the subject is
25 a mammal and especially a primate, for example a
26 human.
27

28 The present invention further
29 provides a method of treating or preventing infection
30 of a plant, said method comprising the step of;
31 - administering, in a therapeutically effective dose,
32 a composition comprising a hollow glucan particle

1 or cell wall particle encapsulating a terpene
2 component to the plant or to soil in proximity to
3 the plant.

4
5 Suitable compositions are those defined in more
6 detail above.

7
8 Terpenes have been shown to eliminate a number of
9 plant pathogens (see WO 03/020024) and, as described
10 in co-pending application US 60/538,627 also
11 effectively kill nematodes which are significant
12 plant parasites. Terpenes alone in suspension or
13 solution, however, are somewhat unstable and degrade
14 rapidly in the soil environment, thus losing
15 efficacy.

16
17 Incorporation of a terpene component in a hollow
18 glucan particle or cell wall particle can reduce the
19 rate of terpene release and degradation, thus
20 increasing the duration of action of the terpene in
21 the soil.

22
23 Suitably the infection of a plant which is to be
24 treated or prevented in the above method is infection
25 by nematodes.

26
27 Other plant infections that may be treated or
28 prevented include fungal plant infections, especially
29 those affecting the surface of a plant. Such
30 infections include downy mildew, powdery mildew or
31 botrytis bunch rot; these infections particularly
32 affect grape vines.

1 In one embodiment, the plant infection may be caused
2 by one or more of the following:

3 *Aspergillius fumigatus*, *Sclerotinta homeocarpa*,
4 *Rhizoctonia solani*, *Colletotrichum graminicola* or
5 *Penicillium* sp.

6

7 An advantage of a terpene based treatment of plants
8 is that it can be applied shortly before harvest.

9

10 Many conventional treatments require an extended
11 period before re-entry to the treated area (generally
12 3 weeks). This means that an outbreak of a plant
13 disease shortly before harvest cannot be treated with
14 conventional treatments as it would then not be
15 possible to harvest the crop at the desired time.

16 The compositions of the present invention can
17 suitably be applied at any time up until harvest, for
18 example 21 days prior to harvest, 14 days prior to
19 harvest, 7 days prior to harvest, or even 3 days or
20 less before harvest.

21

22 Encapsulated terpenes have shown particular efficacy
23 in treating downy mildew, powdery mildew and botrytis
24 bunch rot in grapes, and thus the present invention
25 provides a method of treating or preventing these
26 diseases.

27

28 Prevention of plant infections can be achieved by
29 treating plants with the encapsulated terpenes
30 regularly as a prophylactic measure.

31

1 Suitably the composition of the present invention is
2 applied by spraying. This is particularly suitable
3 for treating a plant disease which affects the
4 surface of a plant. For spraying, a preparation
5 comprising 2 g/l of the composition in water may be
6 used. Concentrations of from 2 to 4 g/l are
7 particularly effective, and concentrations of greater
8 than 4 g/l can be used as required. Obviously it is
9 important that the concentration of the composition
10 used is sufficient to kill or inhibit the disease
11 causing agent, but not so high as to harm the plant
12 being treated.

13
14 When spraying plants a rate of 500 L/Ha or greater is
15 suitable to cover the plants. Preferably a rate of
16 900 L/Ha or greater, more preferably 1200 L/Ha or
17 greater is used to ensure good coverage. Where grape
18 vines are being treated, a rate of 1200 L/Ha has
19 proven suitably effective.

20
21 The composition of the present invention may
22 alternatively be applied via irrigation. This is
23 particularly suitable for treating nematodes or other
24 soil borne pathogens or parasites.

25
26 In a further embodiment the present invention also
27 provides a composition comprising a hollow glucan
28 particle or cell wall particle encapsulating a
29 terpene component for use in the prevention or
30 treatment of an infection in a patient or a plant.
31 Suitable compositions are those defined in more
32 detail above.

In a further embodiment the present invention provides the use of a composition comprising a hollow glucan particle or cell wall particle encapsulating a terpene component in the manufacture of a medicament for the treatment of infection caused by a micro-organism. Suitable compositions are those defined in more detail above.

The present invention further provides the use of a composition comprising a hollow glucan particle or cell wall particle encapsulating a terpene component in the manufacture of a medicament for preventing or treating an infection in a patient, wherein said medicament is suitable for being administered to said patient in a therapeutically effective dose.

The present invention further provides a composition comprising a hollow glucan particle encapsulating a terpene component for use in the prevention or treatment of an infection in a patient or a plant.

The present invention further provides the use of a hollow glucan particle encapsulating a terpene component in the manufacture of a medicament for the treatment of an infection in a plant.

The present invention will now be further described with reference to the following, non-limiting, examples and figures in which:

Fig. 1 represents a light micrograph of empty yeast cell walls;

Fig. 2 represents a light micrograph of yeast cell walls encapsulating L-carvone;

Fig. 3 represents a light micrograph of yeast cell walls encapsulating citral;

Fig. 4 represents a light micrograph of terpene emulsion;

Fig. 5 represents a light micrograph of yeast cell walls in hydrodynamic volume (HV) water;

Fig. 6 represents a light micrograph of yeast cell walls encapsulating terpene in 5 times hydrodynamic volume (HV) of water;

Fig. 7 represents a light micrograph of yeast cell walls encapsulating terpene in HV of water;

Fig. 8 represents a light micrograph of yeast cell walls encapsulating terpene in HV plus 5% of water;

Fig. 9 represents a light micrograph of yeast cell walls encapsulating terpene in HV plus 10% of water;

Fig. 10 represents a light micrograph of yeast cell walls encapsulating terpene in HV plus 20% of water;

1 Fig. 11 represents a light micrograph of yeast cell
2 walls encapsulating terpene in HV plus 30% of water;
3 Fig. 12 represents a light micrograph of yeast cell
4 walls encapsulating terpene in HV plus 40% of water.
5 Fig. 13 represents a light micrograph showing the
6 dispersal of dried hollow glucan particles
7 encapsulating a terpene component and no xanthan gum.
8 Fig. 14 represents a light micrograph as in Fig. 13
9 where 0.07 g of 1% xanthan gum is included.
10 Fig. 15 represents a light micrograph as in Fig. 13
11 where 0.14 g of 1% xanthan gum is included.
12 Fig. 16 represents a light micrograph as in Fig. 13
13 where 0.28 g of 1% xanthan gum is included.
14 Fig. 17 represents a light micrograph as in Fig. 13
15 where 0.55 g of 1% xanthan gum is included.
16 Fig. 18 represents a light micrograph as in Fig. 13
17 where 1.1 g of 1% xanthan gum is included.
18 Fig. 19 represents a light micrograph as in Fig. 13
19 where 2.2 g of 1% xanthan gum is included.
20 Fig. 20 represents a light micrograph as in Fig. 13
21 where 4.4 g of 1% xanthan gum is included.
22 Fig. 21 shows a schematic representation of treatment
23 areas on sites 18 and 20.
24 Fig. 22 shows a schematic representation of treatment
25 areas on sites 18 and 20.
26 Fig. 23 shows a schematic representation of the
27 treatment areas on site 7.
28 Fig. 24 shows a graph showing comparison of
29 encapsulated vs. non-encapsulated terpene
30 formulations.
31

1 The following examples are provided to further enable
2 those of ordinary skill in the art to make or perform
3 the present invention. They are purely exemplary and
4 are not intended to limit the scope of the invention.
5 Unless indicated otherwise, parts are parts by volume
6 or parts by weight, as indicated, temperature is in
7 degrees Celsius (°C) or is at ambient temperature,
8 and pressure is at or near atmospheric. There are
9 numerous variations and combinations of the
10 compositions and conditions for making or using them,
11 e.g., component concentrations, desired solvents,
12 solvent mixtures, temperatures, pressures, and other
13 ranges and conditions that can be used to optimise
14 the results obtained from the described compositions
15 and methods. Only reasonable and routine
16 experimentation will be required to optimise these.

17
18 **Example 1 - Demonstration of Terpene Loading into**
19 **Baker's Yeast Particles and Purified Yeast Glucan**
20 **Particles**

21
22 The following protocol was performed to demonstrate
23 that terpenes would load into yeast cell walls and
24 other hollow glucan particles.

25
26 Emulsions of citral and L-carvone were prepared by
27 mixing 150 µl of the terpene with 100 µl of 10% Tween
28 80 in water and 250 µl of water.

29
30 Baker's yeast particles (YP) or Levacan™ yeast
31 glucan particles (YGP), available from Savory Systems

1 International, Inc., Branchburg, NJ, were mixed with
2 water to form a 250 mg/ml suspension.

3

4 500 µl of the YP or YGP suspension and 250 µl of the
5 terpene emulsion were mixed together and incubated
6 overnight under constant agitation. 500 µl YP or YGP
7 suspension and 500 µl of water were used as a
8 control. The particles were then washed with water
9 until free from external emulsion. The particle
10 preparations were then frozen and lyophilised until
11 dry.

12

13 The particles were then rehydrated and examined under
14 light microscope. The results are shown in Figs. 1
15 to 4.

16

17 Fig. 1 shows spherical structures with a dark area at
18 their centre, these are empty hollow glucan
19 particles. Figs. 2 and 3 shows spherical structures
20 with a swollen appearance with a light coloured
21 interior, these are particles with terpene
22 encapsulated in the central cavity - citral in Fig. 2
23 and L-carvone in Fig. 3. In Figs. 2 and 3 small
24 blobs of free terpene can also be seen, e.g. at the
25 top of Fig. 2, just left of centre. Fig. 4 shows the
26 terpene emulsion as small blebs of terpene suspended
27 in water.

28

29

30

1 Example 2 - Determination of maximal citral and L-
2 carvone loading levels in Baker's Yeast Cell Wall
3 Particles (YP)

4
5 The following protocol was performed to determine the
6 maximal amounts of terpenes that would load into YP.

- 7
8 - L-carvone and citral emulsions were prepared by
9 sonicating 4.5 g of the terpene with 0.3 ml water.
10 - 10% Tween-80 solution was prepared by sonicating
11 4.5 g Tween-80 in 40.5 mls water.
12 - YP suspension was prepared by mixing YP with water
13 to form 20 mg/ml suspension.
14 - Encapsulation reactions were set up as described
15 in Table 1.

16
17 Citral or L-carvone-water emulsion was mixed with YP
18 and Tween 80 surfactant overnight at room
19 temperature. Samples were centrifuged at 14,000 x g
20 for 10 minutes and the appearance of free terpene
21 floating on the aqueous layer was scored. The
22 results are shown in the right hand column labelled
23 free terpene of Table 1.

24
25 The expression "free terpene" refers to the visible
26 presence of terpene in the centrifuged reaction
27 mixture. The absence of free terpene indicates
28 complete absorption of the terpene by the particles.
29 The highest volume of terpene absorbed by the
30 particles, as evidenced by the absence of free
31 terpene, was recorded as the maximal volume of
32 absorbed terpene emulsion.

1 **Table 1**

Tube	20 mg/ml YP	Terpene Emulsion	Vol	10% Tween- 80	Free Terpene
	μ l		μ l	μ l	
1	500	-	-	500	-
2	500	L-carvone	0.5	500	-
3	500	L-carvone	1.65	500	-
4	500	L-carvone	5	495	-
5	500	L-carvone	16.5	483.5	-
6	500	L-carvone	50	450	+
7	500	L-carvone	165	335	+
8	500	L-carvone	500	-	+
9	500	Citral	0.5	500	-
10	500	Citral	1.65	500	-
11	500	Citral	5	495	-
12	500	Citral	16.5	483.5	+/-
13	500	Citral	50	450	+
14	500	Citral	165	335	+
15	500	Citral	500	-	+

2
3 As can be seen from the results, YP is capable of
4 absorbing and encapsulating at least 16.5 μ l of L-
5 carvone terpene emulsion or at least 5 μ l of citral
6 emulsion per 10 mg of YP.

7
8 **Example 3 - Demonstration of improved terpene loading**
9 **with surfactant and determination of optimal Tween-**
10 **80:Terpene ratio**

11
12 The following protocol was performed to demonstrate
13 that the presence of surfactant improves terpene
14 loading and to determine the minimum level of Tween-

1 80 surfactant required for the YP terpene loading
2 reaction.

3

- 4 - L-carvone and citral emulsions were prepared by
5 sonicating 4.5 g of the terpene with 0.3 ml water.
- 6 - 10% Tween-80 solution was prepared by sonicating
7 4.5 g Tween-80 in 40.5 ml water.
- 8 - Baker's YP suspension was prepared by mixing YP
9 with water to form 250 mg/ml suspension.

10

11 Loading reactions were set up as shown in Table 2
12 below.

13

14 Citral or L-carvone-water emulsion was mixed with YP
15 with 0 - 10% v/v Tween 80 surfactant overnight at
16 room temperature. Samples were centrifuged at 14,000
17 x g for 10 minutes and the appearance of free terpene
18 floating on the aqueous layer was scored. The
19 results are shown in the right hand column labelled
20 free terpene of Table 2.

21

22 The expression "free terpene" refers to the visible
23 presence of terpene in the centrifuged reaction
24 mixture. The absence of free terpene indicates
25 complete absorption and encapsulation of the terpene
26 by the YP. The highest volume of terpene absorbed by
27 the YP, as evidenced by the absence of free terpene,
28 was recorded as the maximal volume of absorbed
29 terpene emulsion.

30

31

32

1 **Table 2**

<u>Tube</u>	<u>250</u> <u>mg/ml YP</u>	<u>Terpene</u> <u>Emulsion</u>	<u>Vol</u>	<u>10% Tween-</u> <u>80</u>	<u>Water</u>	<u>Free</u> <u>Terpene</u>
	ml		μ l	μ l	μ l	
1	500	-	-	-	500	-
2	500	L-carvone	150	0	350	S1
3	500	L-carvone	150	5	345	S1
4	500	L-carvone	150	10	340	S1
5	500	L-carvone	150	33	317	S1
6	500	L-carvone	150	100	250	-
7	500	L-carvone	150	200	150	-
8	500	L-carvone	150	350	-	-
9	500	L-carvone	400	0	100	++
10	500	L-carvone	400	5	95	++
11	500	L-carvone	400	10	90	++
12	500	L-carvone	400	33	77	++
13	500	L-carvone	400	100	-	+
14	500	L-carvone	400	20 μ l 100%	30	+
15	500	Citral	113	0	387	+
16	500	Citral	113	5	382	+
17	500	Citral	113	10	377	+
18	500	Citral	113	33	354	S1
19	500	Citral	113	100	287	S1
20	500	Citral	113	200	187	-
21	500	Citral	113	350	37	-
22	500	Citral	250	0	250	++
23	500	Citral	250	5	245	++
24	500	Citral	250	10	240	++
25	500	Citral	250	33	217	+
26	500	Citral	250	100	150	+
27	500	Citral	250	20 μ l 100%	230	+

1 S1 = slight

2

3 As can be seen from the results a Tween-80
4 concentration of 1% (i.e. 100 µl of 10 % Tween-80 in
5 1000 µl of reaction mixture) is sufficient to allow
6 complete uptake of the terpene in the above reaction.
7 A 2% Tween-80 causes no improvement in results,
8 whereas with a 0.33% concentration free terpene was
9 observed. This indicates that:

- 10 a) Terpenes are absorbed into YP particles in the
11 absence of a surfactant, but the presence of
12 surfactant significantly increases terpene
13 absorption.
- 14 b) A Tween-80 concentration of around 1% is optimum
15 for YP loading as it ensures proper loading
16 whilst maximising the terpene payload of the YP
17 particles.

18

19 **Example 4 - Determination of maximal terpene loading**
20 **and encapsulation at high Baker's Yeast Cell Wall**
21 **Particles (YP) levels**

22

23 The following protocol was performed to determine the
24 maximal amounts of terpenes that would load into YP
25 at high YP levels.

26

- 27 - L-carvone and citral emulsions were prepared by
28 sonicating 4.5 g of the terpene with 3 ml 1%
29 Tween.
- 30 - 5% Tween-80 solution was prepared by sonicating
31 0.5 g Tween-80 in 9.5 ml water.

- 1 - YP suspension was prepared by mixing YP with water
2 to form 250 mg/ml suspension.
3 - Encapsulation reactions were set up as shown in
4 Table 3.

5
6 Citral or L-carvone-water emulsion was mixed with YP
7 and Tween 80 surfactant overnight at room
8 temperature. Samples were centrifuged at 14,000 x g
9 for 10 minutes and the appearance of free terpene
10 floating on the aqueous layer was scored. The
11 results are shown in the right hand column labelled
12 free terpene of Table 3.

13
14 The expression "free terpene" refers to the visible
15 presence of terpene in the centrifuged reaction
16 mixture. The absence of free terpene indicates
17 complete absorption of the terpene by the YP. The
18 highest volume of terpene absorbed by the YP, as
19 evidenced by the absence of free terpene, was
20 recorded as the maximal volume of absorbed terpene
21 emulsion.

22
23
24
25
26
27
28
29
30
31
32

1 **Table 3**

<u>Tube</u>	<u>250</u> <u>mg/ml YP</u>	<u>Terpene</u> <u>Emulsion</u>	<u>Vol</u>	<u>1% Tween-</u> <u>80</u>	<u>Free</u> <u>Terpene</u>
	μ l		μ l	μ l	
1	500	-	-	500	-
2	500	L-carvone	15	485	-
3	500	L-carvone	37.5	462.5	-
4	500	L-carvone	75	425	-
5	500	L-carvone	112.5	387.5	-
6	500	L-carvone	150	350	Sl +
7	500	L-carvone	225	275	+
8	500	L-carvone	450	50	+
9	500	Citral	15	485	-
10	500	Citral	37.5	462.5	-
11	500	Citral	75	425	-
12	500	Citral	112.5	387.5	Sl +
13	500	Citral	150	350	+
14	500	Citral	225	275	+
15	500	Citral	450	50	+

2

3 As can be seen from the results in Table 3, YP is
4 capable of absorbing and encapsulating terpenes at
5 high YP concentration. YP absorbed and encapsulated
6 at least 112.5 μ l of L-carvone terpene emulsion or at
7 least 75 μ l of citral emulsion per 125 mg of YP.
8 This demonstrates that the terpene encapsulation
9 reaction is independent of YP concentration within
10 the ranges tested.

11

12

1 Example 5 - Screen commercially available particles
2 for terpene absorption

3

4 The following protocol was performed to analyse the
5 loading properties of different types of particles.

6 The particles studied were Baker's Yeast Cell Wall
7 Particles (Sigma Chemical Corp., St. Louis, MO),
8 NutrexTM Walls (Sensient Technologies, Milwaukee,
9 WI), SAF-MannanTM (SAF Agri, Minneapolis, MN),
10 Nutricept WallsTM (Nutricepts Inc., Burnsville, MN),
11 LevacanTM (Savory Systems International, Inc.,
12 Branchburg, NJ) and WGPTM (Alpha-beta Technology,
13 Inc. Worcester, MA).

14

15 L-carvone and citral emulsions were prepared by
16 sonicating 7 g terpene + 3 ml 3.3% Tween-80.

17

18 Table 4 below compares the purity with the number of
19 yeast particles per mg and the packed solids
20 weight/volume ratio.

21

22

23

24

25

26

27

28

29

30

31

32

1 **Table 4**

<u>Yeast Particle</u>	<u>Purity</u>	<u>No. particles/mg</u>	<u>Mg particles/ml</u>
	<u>% Beta 1,3-glucan</u>		
Bakers	11.2	4×10^7	250
Nutrex	24.5	1.7×10^8	58.8
SAF Mannan	33.4	2.4×10^8	41.7
Nutraceuticals	55.7	5.2×10^8	37
Levacan	74.6	1×10^8	19.2
WGP	82.1	3.5×10^8	10

2

3 From Table 4 it can be concluded that the number of
4 particles per mg is inversely proportional to purity.
5 Thus the number of particles per mg of WGP is almost
6 10-fold higher than Baker's YP.

7

8 The YP suspensions were prepared as follows:

9

- 10 - Baker's yeast cell wall particle suspension (YP)
- 11 was prepared by mixing 250 mg YP / ml 1% Tween 80.
- 12 - Nutrex suspension was prepared by mixing 163 mg
- 13 Nutrex YGP / ml 1% Tween 80.
- 14 - SAF Mannan suspension was prepared by mixing 234
- 15 mg Biospringer YGP / ml 1% Tween 80.
- 16 - Nutricepts suspension was prepared by mixing 99
- 17 mg Nutricepts YGP / ml 1% Tween 80.
- 18 - Levacan suspension was prepared by mixing 217 mg
- 19 Lev YGP / ml 1% Tween 80.
- 20 - WGP suspension was prepared by mixing 121 mg WGP
- 21 YGP / ml 1% Tween 80.

22

1 The packed volume of the above particles is identical
2 which means that equal numbers of particles were
3 assayed.

4
5 Loading reactions were set up as shown in Table 5 and
6 left to incubate overnight. Samples were centrifuged
7 at 14,000 x g for 10 minutes and the appearance of
8 free terpene floating on the aqueous layer and the
9 color of the encapsulated terpenes in the pellet was
10 scored. The results are shown in the two right hand
11 columns of Table 5. The highest volume of terpene
12 absorbed by particles as evidenced by the absence of
13 free terpene was recorded as the volume of absorbed
14 terpene emulsion.

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1 **Table 5**

Tube	Particle	conc	μ l	Terpene	Vol	1% Tween		Free	Colour
		mg/ml		Emulsion		80	μ l	Terpene	
1	Baker's	250	500	L-carvone	125	375		-	W
2	Nutrex	163	500	L-carvone	125	375		-	W
3	SAF Mannan	234	500	L-carvone	125	375		-	W
4	Nutricepts	99	500	L-carvone	125	375		+	W
5	Levacan	217	500	L-carvone	125	375		+	W
6	WGP	121	500	L-carvone	125	375		+	W
7	Baker's	250	500	Citral	100	375		-	Y
8	Nutrex	163	500	Citral	100	375		-	Y
9	SAF Mannan	234	500	Citral	100	375		-	W
10	Nutricepts	99	500	Citral	100	375		+	Y
11	Levacan	217	500	Citral	100	375		+	int
12	WGP	121	500	Citral	100	375		+	int
13	-	-	-	L-carvone	125	875		+	-
14	-	-	-	Citral	100	900		+	Y

2 W = white; Y = yellow; sl = slight; int =
3 intermediate

4

5 From the results the following conclusions were
6 reached:

- 7 - Purified particles with a low lipid content were
8 less effective at absorbing terpenes.
9 - Less pure particles were more effective at
10 absorbing terpenes.
11 - Yellow degradation product of citral was not
12 formed when encapsulated in SAF-MannanTM.
13 - Based on qualitative loading at the single terpene
14 level tested, SAF MannanTM appears to be best,
15 NutrexTM second and Baker's third.

16

1 Example 6 - Kinetics of terpene loading into various
2 types of particles and different incubation
3 temperatures.

4
5 The following protocol was adopted to compare the
6 loading kinetics of various types of yeast particles.

7
8 L-carvone and citral emulsions were prepared by
9 sonicating 7 g terpene with 3 ml 3.3% Tween-80.

10
11 1% Tween-80 solution was prepared by sonicating 1 ml
12 10% Tween-80 in 10 ml water.

13
14 - Baker's YP was prepared by mixing 5 g of bakers YP
15 in 20 ml 1% Tween-80.

16 - NutrexTM YGP suspension was prepared by mixing 2
17 g NutrexTM YGP in 20 ml 1% Tween-80.

18 - SAF MannanTM suspension was prepared by mixing 2 g
19 SAF MannanTM in 20 ml 1% Tween-80.

20
21 Loading reactions were set up as shown in Table 6.

22
23 The reactions were incubated for 1, 3, 6, 9 and 24
24 hours at room temperature or 37 °C. After incubation
25 samples were centrifuged at 14,000 x g for 10 minutes
26 and the appearance of free terpene floating on the
27 aqueous layer was scored. The results are shown in
28 the two right hand columns of Table 6. The highest
29 volume of terpene absorbed by the particles as
30 evidenced by the absence of free terpene was recorded
31 as the volume of absorbed terpene emulsion. Colour
32 of the encapsulated pellet was scored at 24 hours.

Table 6

Tube	T °C	Particle	conc mg/ml	μ l	Terpene Emulsion	Vol μ l	1% Tween-80	Free Terpene (hr)					Color
								1	3	6	9	24	
1	Rt	Bakers	250	3500	L-carvone	788	2712	+	-	-	-	-	W
2	37	Bakers	250	3500	L-carvone	788	2712	+	-	-	-	-	W
3	Rt	Nutrex	100	3500	L-carvone	1050	2450	+	-	-	-	-	W
4	37	Nutrex	100	3500	L-carvone	1050	2450	+	-	-	-	-	W
5	Rt	SAF	100	3500	L-carvone	1050	2450	<+	-	-	-	-	W
6	37	SAF	100	3500	L-carvone	1050	2450	<+	-	-	-	-	W
7	Rt	Bakers	250	3500	Citral	525	2975	+	-	-	-	-	Y
8	37	Bakers	250	3500	Citral	525	2975	+	-	-	-	-	VY
9	Rt	Nutrex	100	3500	Citral	788	2712	+	-	-	-	-	Y
10	37	Nutrex	100	3500	Citral	788	2712	+	-	-	-	-	VY
11	Rt	SAF	100	3500	Citral	788	2712	+	-	-	-	-	W
12	37	SAF	100	3500	Citral	788	2712	+	-	-	-	-	W

White, W; Yellow, Y; Very Yellow, VY; Room Temperature, Rt

1

2

3

4

- 1 From the results shown in Table 6 and other
2 observations the following conclusions can be made:
- 3 • Terpene loading reaction takes between 1 and 3
4 hours.
 - 5 • Terpene loading occurs faster at 37 °C than at
6 room temperature.
 - 7 • SAF MannanTM appears to be preferable particles for
8 two reasons:
 - 9 - Faster and more complete uptake of both
10 terpenes.
 - 11 - Citral remains stable when loaded as
12 evidenced by the absence of yellow colour,
13 characteristic of citral degradation, after
14 24 hours at 37 °C.

15
16 **Example 7 - Screen a range of single terpenes and**
17 **terpene combinations for particle loading**

18
19 The following protocol was adopted to compare the
20 loading efficiency of Baker's YP versus SAF MannanTM.

21
22 Terpene emulsions were prepared as follows:

- 23 - L-carvone - 4.5 g L-carvone in 1.5 ml 3.3% Tween-
24 80.
- 25 - Citral - 4.5 g citral in 1.5 ml 3.3% Tween-80.
- 26 - Thymol/L-carvone mixture (T/L)- 2.25 g thymol and
27 2.25 g L-carvone in 1.5 ml 3.3% Tween-80.
- 28 - Eugenol - 4.5 g eugenol in 1.5 ml 3.3% Tween-80.
- 29 - Geraniol - 4.5 g geraniol in 1.5 ml 3.3% Tween-80.

1 - Citral/L-carvone/Eugenol mixture (C/L/E) - 1.5 g
 2 citral, 1.5 g L-carvone, 1.5 g eugenol in in 1.5
 3 ml 3.3% Tween-80.

4
 5 Emulsions composed of terpene : water : surfactant
 6 ratio of 0.75:0.3:0.05 were used for these
 7 experiments.

8
 9 Increasing volumes of terpene emulsion were mixed
 10 with 250 mg/ml Baker's YP or 250 mg/ml SAF MannanTM
 11 overnight at room temperature as shown in Tables 7
 12 and 8. Samples were centrifuged at 14,000 x g for 10
 13 minutes and the appearance of free terpene floating
 14 on the aqueous layer was scored. The highest volume
 15 of terpene emulsion absorbed by Baker's YP or SAF
 16 MannanTM as evidenced by the absence of free terpene
 17 was recorded as the volume of absorbed terpene
 18 emulsion. Colour of encapsulated terpenes in the
 19 pellet was recorded. The results in Tables 7 and 8
 20 show that all single and terpene combinations were
 21 efficiently loaded into both Baker's YP or SAF Mannan
 22 particles.

23
 24 **Table 7** - Evaluation of Baker's YP Loading of
 25 Different Terpenes and Terpene Mixtures.

<u>Tube</u>	<u>Baker</u> <u>(μl)</u>	<u>Terpene</u> <u>Emulsion</u>	<u>Vol</u> <u>(μl)</u>	<u>1% Tween-</u> <u>80 (μl)</u>	<u>Free</u> <u>Terpene</u>	<u>Colour</u>
1	500	-	-	500	-	W
2	500	L-carvone	15	485	-	W
3	500	L-carvone	37.5	462.5	-	W
4	500	L-carvone	7	425	+/-	W

48

5	500	L-carvone	112.5	387.5	+/-	W
6	500	L-carvone	150	350	+	W
7	500	L-carvone	225	275	+	W
8	500	L-carvone	450	50	++	W
9	500	Citral	15	485	-	Y
10	500	Citral	37.5	462.5	-	Y
11	500	Citral	75	425	-	Y
12	500	Citral	112.5	387.5	+/-	Y
13	500	Citral	150	350	+	Y
14	500	Citral	225	275	+	Y
15	500	Citral	450	50	+	Y
16	500	T/L	15	485	-	W
17	500	T/L	37.5	462.5	-	W
18	500	T/L	75	425	-	W
19	500	T/L	112.5	387.5	+/-	W
20	500	T/L	150	350	+	W
21	500	T/L	225	275	+	W
22	500	T/L	450	50	+	W
23	500	Eugenol	15	485	-	W
24	500	Eugenol	37.5	462.5	-	W
25	500	Eugenol	75	425	-	W
26	500	Eugenol	112.5	387.5	+/-	W
27	500	Eugenol	150	350	+	W
28	500	Eugenol	225	275	+	W
29	500	Eugenol	450	50	+	W
30	500	Geraniol	15	485	-	W
31	500	Geraniol	37.5	462.5	-	W
32	500	Geraniol	75	425	-	W
33	500	Geraniol	112.5	387.5	+	W
34	500	Geraniol	150	350	+	W
35	500	Geraniol	225	275	+	W

49

36	500	Geraniol	450	50	+	W
37	500	C/L/E	15	485	-	Y
38	500	C/L/E	37.5	462.5	-	Y
39	500	C/L/E	75	425	-	Y
40	500	C/L/E	112.5	387.5	+/-	Y
41	500	C/L/E	150	350	+	Y
42	500	C/L/E	225	275	+	Y
43	500	C/L/E	450	50	+	Y

1

2 **Table 8** - Evaluation of SAF Mannan Loading of
3 Different Terpenes and Terpene Mixtures.

4

<u>Tube</u>	<u>SAF</u> <u>(μl)</u>	<u>Terpene</u> <u>Emulsion</u>	<u>Vol</u>	<u>1% Tween-</u> <u>80 (μl)</u>	<u>Free</u> <u>Terpene</u>	<u>Colour</u>
1	500	-	-	500	-	W
2	500	L-carvone	15	485	-	W
3	500	L-carvone	37.5	462.5	-	W
4	500	L-carvone	75	425	-	W
5	500	L-carvone	112.5	387.5	-	W
6	500	L-carvone	150	350	+/-	W
7	500	L-carvone	225	275	+/-	W
8	500	L-carvone	450	50	+	W
9	500	Citral	15	485	-	W
10	500	Citral	37.5	462.5	-	W
11	500	Citral	75 μ l	425	-	W
12	500	Citral	112.5	387.5	-	W
13	500	Citral	150	350	+/- Inverted	W
14	500	Citral	225	275	+ Inverted	W
15	500	Citral	450	50	+ Inverted	W
16	500	T/L	15	485	-	W

50

17	500	T/L	37.5	462.5	-	W
18	500	T/L	75	425	-	W
19	500	T/L	112.5	387.5	-	W
20	500	T/L	150	350	+/-	W
21	500	T/L	225	275	+	W
22	500	T/L	450	50	+	W
23	500	Eugenol	15	485	-	W
24	500	Eugenol	37.5	462.5	-	W
25	500	Eugenol	75	425	-	W
26	500	Eugenol	112.5	387.5	+/-	W
27	500	Eugenol	150	350	+	W
28	500	Eugenol	225	275	+	W
29	500	Eugenol	450	50	+	W
30	500	Geraniol	15	485	-	W
31	500	Geraniol	37.5	462.5	-	W
32	500	Geraniol	75	425	-	W
33	500	Geraniol	112.5	387.5	-	W
34	500	Geraniol	150	350	-	W
35	500	Geraniol	225	275	- Inverted	W
36	500	Geraniol	450	50	+ Inverted	W
37	500	C/L/E	15	485	-	W
38	500	C/L/E	37.5	462.5	-	W
39	500	C/L/E	75	425	-	W
40	500	C/L/E	112.5	387.5	-	W
41	500	C/L/E	150	350	-	W
42	500	C/L/E	225	275	+/-	W
43	500	C/L/E	450	50	+	W

1

2 Inverted = Phase Inverted - solids floating on top

3 - no free oil; W = white; Y = yellow.

1 From the results the following observations were
2 made:

- 3 - All terpenes appeared to load into Baker's YP and
4 SAF Mannan.
- 5 - SAF Mannan has a higher terpene loading capacity
6 than bakers YP.
- 7 - The two and three way mixtures of terpenes also
8 appear to efficiently load.
- 9 - The terpene Eugenol appears to have a higher
10 density than the particles and water as it was
11 found associated with the pellet.
- 12 - For the SAF Mannan, the higher load levels and
13 lighter particles resulted in loaded particles
14 floating on the surface of the aqueous layer for
15 citral and geraniol.
- 16 - Citral was protected from oxidation by the SAF
17 Mannan but not by the Baker's YP.

18

19 The approximate maximal loading for each particle
20 type was determined and is shown in Tables 9 and 10
21 below. Percentage loaded represents a ratio of the
22 amount of terpene loaded to the amount of particle
23 present (weight for weight).

24

25

26

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28

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32

1 **Table 9** - Maximal terpene loading in Baker's YP.

2

Terpene	Vol. Loaded μ l	% Loaded w/w
L-carvone	37.5	33.3
Citral	75	67%
Thymol/L-carvone 1:1	75	67%
Eugenol	75	67%
Geraniol	75	67%
Citral/L-carvone/ Eugenol (1:1:1)	75	67%

3

4 **Table 10** - Maximal terpene loading in SAF Mannan.

5

Terpene	Vol. loaded μ l	% Loaded w/w
L-carvone	112.5	100%
Citral	150	133%
Thymol/L-carvone 1:1	112.5	100%
Eugenol	112.5	100%
Geraniol	150	133%
Citral/L-carvone/ Eugenol (1:1:1)	150	133%

6

7 **Example 8 - Evaluation of Terpene stability in**
8 **aqueous emulsions and encapsulated terpene**
9 **formulations**

10

11 Terpene stability was assessed by the observation of
12 citral formulations for the formation of a yellow
13 colored oxidation product. As noted in the right
14 hand column in Tables 5-8 citral emulsions and citral
15 encapsulated Bakers YP turned a progressively
16 increasing yellow color over time. However, citral

1 encapsulation in SAF MannanTM increased citral
2 stability as evidenced by a reduction or absence of
3 yellow color over time.

4

5 **Example 9 - Loading of Terpenes in minimal water**

6

7 The following protocol was carried out to evaluate
8 the possibility that terpene loading and
9 encapsulation into YP could be carried out at a very
10 high Yeast Particles (YP) solids level to allow for
11 direct extrusion of the loaded formulation into a
12 fluidised bed drier. The minimal amount of water to
13 completely hydrate the SAF MannanTM particles was
14 determined to be 3.53 g water per g solids. This
15 defines the hydrodynamic volume (HV) or water
16 absorptive capacity of the particles. At this level
17 of water the hydrated particles have a consistency of
18 a stiff dough which is thixotropic, i.e. shear
19 thinning like mayonnaise. Addition of water up to 40
20 % above the HV results in a thick flowable paste.
21 The standard reaction that has been used in the above
22 examples was carried out at 3 X HV water.

23

24 A series of terpene (L-carvone) loading reactions
25 were carried out keeping the ratio of
26 particle:terpene:Tween (1: 0.44:0.04) constant and
27 varying the amount of water in the system from the HV
28 (3.53 g) to HV + 40% water (4.92 g). Controls were
29 the standard loading system which uses 3 X HV water,
30 particles only and terpene only reactions. Following
31 overnight incubation samples of the mixtures were
32 evaluated microscopically for free terpene and

1 evidence of terpene uptake into the particles and for
 2 material flow characteristics by assessing flow in
 3 inverted tubes over 15 minutes. In addition, the
 4 presence of free oil was assessed by hydrating the
 5 reaction mixture with 5 X HV, vortexing to obtain a
 6 complete dispersion of particles and centrifugation
 7 to sediment the particle encapsulated terpene. The
 8 results are shown in Table 11 and Figs. 7 to 12.
 9 Figs. 7 to 12 show the loading results of the
 10 following tubes:

- 11 - Fig. 7 - Tube 3
- 12 - Fig. 8 - Tube 5
- 13 - Fig. 9 - Tube 6
- 14 - Fig. 10 - Tube 8
- 15 - Fig. 11 - Tube 10
- 16 - Fig. 12 - Tube 11

17
 18 **Table 11**

<u>Tube</u>	<u>SAF</u>	<u>Terpene</u>	<u>Weight</u>	<u>Water</u>	<u>Free</u>	<u>Flow</u>
	<u>g</u>	<u>Emulsion</u>	<u>(g)</u>	<u>(g)</u>	<u>Terpene</u>	
1	-	L-carvone	4.64	4.5	+	+
2	1	-	-	8.0	-	+
3	1	L-carvone	4.64	4.5	-	+
4	1	L-carvone	4.64	-	-	-
5	1	L-carvone	4.64	0.17	-	-
6	1	L-carvone	4.64	0.35	-	-
7	1	L-carvone	4.64	0.52	-	S1
8	1	L-carvone	4.64	0.7	-	Mod
9	1	L-carvone	4.64	0.87	-	High
10	1	L-carvone	4.64	1.05	-	High
11	1	L-carvone	4.64	1.39	-	High

1 The results shown in Table 11 and Figs. 7 to 12
2 demonstrate that terpene loading and encapsulation
3 into the particles occurred at all water ratios
4 evaluated. Surprisingly, equivalent loading occurred
5 even when the loading reaction was taking place in a
6 reaction with the consistency of a stiff dough using
7 the minimal amount of water to hydrate the particles.
8 The absence of free terpene was observed
9 microscopically (Figs. 7 to 12) and in the low level
10 of terpene in the supernatants, as evidenced by a
11 marked reduction in the turbidity of the supernatant
12 compared to the terpene only control.

13
14 These results extend our understanding of the
15 conditions to load terpenes into hollow glucan
16 particles. The flexibility to use a minimal volume
17 of water to hydrate the particles during the loading
18 process will allow loading of the terpenes under
19 conditions where the reaction mixture is a malleable
20 dough-like consistency using standard food-grade
21 swept surface dough mixers. The consistency of the
22 final high solids terpene loaded mixture is suitable
23 for direct extrusion to form noodles and pellets for
24 fluidised bed drying.

25
26 Suitable facilities to scale up production in this
27 manner would require:

- 28 - Gaulin homogeniser, or equivalent to produce
- 29 stable terpene emulsion.
- 30 - Swept surface dough mixing tank.
- 31 - Extruder.
- 32 - Fluidised bed drier.

1 Example 10 - Evaluation of an interstitial
2 hydrocolloid agent to aid dispersion in dried hollow
3 glucan particles encapsulating a terpene component
4 dispersion when re-hydrated.

5
6 The following protocol was adopted to evaluate the
7 effect of an interstitial hydrocolloid to increase
8 dried hollow glucan particle encapsulated terpene
9 formulations to disperse when hydrated.

- 10 - SAF MannanTM particles
11 - 0.1% Tween 80
12 - L-carvone
13 - Xanthan Gum - 1% w/v in 0.1% Tween 80

14
15 The effect of increasing xanthan gum levels on dry
16 hollow glucan particle encapsulated L-carvone
17 dispersion in water was assessed by loading L-carvone
18 into SAF Mannan by incubating 1.1 g of an L-carvone
19 emulsion (L-carvone : water : surfactant ratio of
20 0.75:0.3:0.05) with 1 g SAF Mannan and 4.4 g 0.1%
21 Tween 80 containing 0 - 1% xanthan gum as shown in
22 Table 12.

23
24
25
26
27
28
29

1 **Table 12**

<u>Tube</u>	<u>SAF</u> <u>g</u>	<u>L-carvone</u> <u>Emulsion</u> <u>(g)</u>	<u>0.1%</u> <u>Tween-80</u> <u>(g)</u>	<u>1%</u> <u>Xanthan</u> <u>(g)</u>	<u>Visual</u> <u>Observations</u>
1	1	1.1	4.4	0	Large non-uniform clumps
2	1	1.1	4.33	0.07	Uniform suspension
3	1	1.1	4.26	0.14	Uniform suspension
4	1	1.1	4.12	0.28	Uniform suspension
5	1	1.1	3.85	0.55	Uniform suspension
6	1	1.1	3.3	1.1	Finer Uniform suspension
7	1	1.1	2.2	2.2	Finer Uniform suspension
8	1	1.1	0	4.4	Finer Uniform suspension

2

3 The results in Table 12 and Figs 13 to 20 demonstrate
4 that the inclusion of a high molecular weight
5 hydrocolloid during the drying of the particle
6 encapsulated terpene aids in the hydration and
7 dispersion of the microparticles into a uniform
8 suspension. Other examples of such hydrocolloid
9 agents are maltodextrin, alginates, or the like.

10

11 It may also be worthwhile to include a pellet coating
12 to increase the stability of the loaded terpenes, and
13 to provide a sustained release of terpene.

Example 11 - Evaluation of minimum inhibitory concentration (MIC) of terpene emulsions, fresh Baker's YP and SAF Mannan encapsulated terpenes and freeze-dried Baker's YP and SAF Mannan encapsulated terpenes against *S. aureus*

The results of a protocol performed to compare the MIC of fresh versus freeze dried hollow glucan particle encapsulated terpene formulations are shown below in Table 13. A simple terpene emulsion was also tested and the results are shown for comparison.

Table 13 MIC µg/ml terpene

<u>Terpene</u>	<u>Emulsion</u>	<u>Bakers</u>		<u>SAF Mannan</u>	
		<u>Fresh</u>	<u>Freeze Dried</u>	<u>Fresh</u>	<u>Freeze Dried</u>
L-carvone	3.75	0.1	>0.04	0.01	>0.02
Citral	0.94	0.01	0.05	0.01	>0.03
L-carvone/ Thymol	0.23	0.01	0.03	0.01	0.05
Eugenol	0.12	0.03	0.05	0.01	0.05
Geraniol	0.47	0.03	0.06	0.02	>0.03
L-carvone/ Citral/Eugenol	0.23	0.03	0.06	0.02	0.05

14

15 The conclusions taken from the above results were:

- 16 - Terpene loading into hollow glucan particles
- 17 appears to enhance terpene MIC. Generally the
- 18 fresh terpene emulsions are ~ 4 - 375 fold less
- 19 potent than the encapsulated formulations
- 20 - Terpenes loaded in SAF MannanTM perform slightly
- 21 better than Baker's YP.

- 1 - Freshly loaded terpene compositions perform
2 slightly better than freeze dried compositions
3 (there may be some volatilisation of terpenes from
4 dry compositions during freeze drying).
5 - Terpenes in aqueous emulsions are stable for at
6 least 3 weeks.

7

8 **Example 12 - Efficacy of encapsulated terpenes at**
9 **pilot plant scale against *S. aureus*.**

10

11 Anti-microbial assays were carried out with
12 encapsulated terpenes and mixtures produced at the
13 pilot plant scales against *S. aureus*. Both the fresh
14 and freeze dried encapsulated terpene samples
15 containing materials demonstrated strong anti-
16 microbial activities. The results are summarised in
17 Table 14 below.

18

19 Terpenes were encapsulated in SAF-MannanTM at a 2.5
20 Kg scale. A mixture of three terpenes (Geraniol, 275
21 g; Eugenol, 385 g; and thymol, 440 gram was dissolved
22 and homogenized with 100 g Tween-80 and 8L of water.
23 SAF-MannanTM (2.5 Kg) was added to form a homogenous
24 suspension. The suspension was passed through a
25 Gaulin homogenizer to reduce particle size and the
26 homogenate was incubated overnight at room
27 temperature. A sample of the encapsulated terpene
28 was removed and stored at room temperature. The
29 remaining encapsulated terpene was then frozen in
30 trays and freeze dried. The freeze dried
31 encapsulated terpene powder was ground and stored at
32 room temperature.

33

1 **Table 14**

Material	MIC (ppm)
<i>Staphylococcus aureus</i> assays	
YGP empty shell control	>2500
Pilot Plant - Fresh	100
Pilot Plant - Freeze dried	100

2

3 At the pilot plant scale both the fresh and freeze
4 dried samples were equally potent on a w/w terpene
5 basis.

6

7 Based on the large scale preparation results, the
8 predicted effective dose of the freeze dried
9 formulation against *S. aureus* is 200 ppm (the
10 formulation contains ~50% terpene w/w) or 0.2 g/L
11 water.

12

13 **Example 13 - Efficacy of encapsulated terpenes**
14 **against Mycobacterium**

15

16 Terpene emulsions were prepared as follows:

- 17 - Citral - 4.5 g citral in 1.5 ml 3.3% Tween-80.
- 18 - L-carvone/eugenol - 2.25 g L-carvone and 2.25 g
19 Eugenol in 1.5 ml 3.3% Tween-80.
- 20 - Eugenol - 4.5 g eugenol in 1.5 ml 3.3% Tween-80.
- 21 - Geraniol - 4.5 g geraniol in 1.5 ml 3.3% Tween-80.
- 22 - Geraniol/thymol mixture - 2.25 g geraniol and 2.25
23 g thymol in 1.5 ml 3.3% Tween-80.
- 24 - Control emulsion - 6 ml 1% Tween-80.

25

61

1 SAF-MannanTM (2.5 g) was mixed with 3 ml of each
2 emulsion and 7 ml of 1% Tween 80 and incubated
3 overnight to encapsulate the terpenes and terpene
4 mixtures. The encapsulated terpene formulations were
5 frozen and freeze dried and the powders ground to a
6 fine powder. Suspensions of encapsulated terpenes
7 (25 mg/ml) and unencapsulated terpene emulsions were
8 assayed for antibacterial activity against
9 *Mycobacterium*. The results are set out in Table 15

10

11 **Table 15**

Material	MIC (ppm)
<i>Mycobacterial assays</i>	
YGP Citral FD	250
YGP L-Carvone/Eugenol FD	500
YGP Eugenol FD	500
YGP Geraniol FD	125
YGP Geraniol/Thymol FD	62.5
Control Emulsion	>1000
Citral Emulsion	35
L-carvone/Eugenol Emulsion	53
Eugenol Emulsion	105
Gernaniol Emulsion	70
Geraniol/Thymol Emulsion	53

12 FD = (Freeze Dried)

13

14 **Example 14 - Nematocidal Activity of Encapsulated**
15 **Terpenes**

16

17 Preparations of yeast cell walls encapsulating citral
18 were prepared according to the procedures described
19 above. The hollow glucan particles contained 17.5%

1 citral, and the particles were present at in the test
2 preparations at a concentration of 1000 ppm. This
3 means that terpenes were effectively present at a
4 concentration of 175 ppm.

5
6 1.0 ml of the test preparations was added to 0.1 to
7 0.15 ml of water containing root-knot nematodes. 1.0
8 water was added to the nematodes as the control.

9
10 Observations were made as previously described and
11 the kill rate assessed (i.e. percentage dead) after
12 24 and 48 hrs. The results shown below in Table 16
13 are an average of 2 sets of results.

14
15 **Table 16** - Nematicidal activity of encapsulated
16 terpene solution (17.5 % citral @ 1000ppm)

17

Time	Kill Rate	
	Test	Control
24 h	45	17
48 h	56	21

18
19 The results demonstrate that hollow glucan particles
20 encapsulating terpenes are effective at killing root-
21 knot nematodes at a particle concentration of 1000
22 ppm, which corresponds to a citral concentration of
23 only 175 ppm.

24
25 Thus hollow glucan particles encapsulating terpenes
26 appear to be as effective as terpenes in solution or
27 with surfactant as nematicides. The nematicidal
28 activity is retained despite the terpene being

1 encapsulated within the particle. It can be expected
2 that higher concentrations of terpenes within the
3 hollow glucan particles, or higher concentrations of
4 the particles would result in an even higher kill
5 rate, as is the case for terpenes in solution or with
6 surfactant.

7

8 **Example 15 - Fungicidal Properties of Encapsulated and**
9 **Non-Encapsulated Terpenes**

10

11 The following protocols were carried out to assess
12 the fungicidal properties of various terpene
13 combinations, and to compare the efficacy of
14 encapsulated and non-encapsulated compositions.

15

16 **Assessment of anti-fungal properties of different**
17 **terpene formulation**

18

19 A microtitre plate assay was used to assess the
20 minimum inhibitory concentration (MIC) of a range of
21 terpene compounds against different pathogenic
22 organisms. The assay used for each organism is
23 described in detail later but important general
24 features are as follows.

25

26 The assay uses two incubation periods to distinguish
27 between static (growth inhibition) and cidal
28 (killing) activities. The first incubation period
29 allows assessment of growth inhibition, but cannot
30 distinguish between merely prevention of growth and
31 killing of the cells. The purpose of the second
32 incubation period is to allow sufficient time and

1 nutrients for any dormant or inhibited cells that
2 survive terpene exposure to proliferate. Any cells
3 that were inhibited by fungistatic effects should
4 respond and grow during the second incubation period,
5 whereas cells that were killed by exposure to
6 terpenes will not grow in the fresh medium.

7
8 Initial screening experiments were carried out using
9 a total of 31 different terpene formulations (Table
10 17). These experiments were repeated using a subset
11 of strongly active terpene formulations (Table 18).

12
13 A combination of the terpenes geraniol, eugenol and
14 thymol in a ratio of 2:1:2 encapsulated within glucan
15 particles was also tested; this sample is referred to
16 as YP-GET. A non-encapsulated geraniol, eugenol and
17 thymol combination in the same ratio was also tested
18 for comparison with the encapsulated form.

19
20 MIC assay using *Saccharomyces cerevisiae*

21
22 *S. cerevisiae* (5×10^5 cells/mL in YPD growth medium)
23 were added to each well of a 96-well microtitre plate
24 in 100 μ L aliquots. At least one column per plate
25 was designated as a cell-only control and no terpene
26 was added to these wells. Aliquots (100 μ L) of
27 different terpene formulations were added to the
28 first row of the remaining columns, and serial 2-fold
29 dilutions were performed by transferring 100 μ L from
30 one row to the next a total of 7 times. Finally, 100
31 μ L was discarded from the last row in order to ensure

65

1 that all wells contained the same volume. Microtitre
2 plates were incubated statically overnight at 30°C.

3

4 Following incubation, plates were scored for
5 inhibition of growth (evidenced by a lack of
6 turbidity). Growth inhibition ($\geq 75\%$) was visually
7 confirmed by microscopy.

8

9 Once the MIC had been determined for each
10 formulation, the microtitre plates were centrifuged
11 and the spent medium was removed from non-turbid
12 wells. The cells were resuspended in fresh medium
13 (100 μL) and the plates were re-incubated overnight
14 at 30°C. Assessment of growth inhibition was
15 performed as before.

16

17 MIC assay using a mixed inoculum

18

19 The different terpene formulations were serially
20 diluted in the 96-well microtitre plate as described
21 for *S. cerevisiae*. Molten YPD agar was then added to
22 the wells, together with 5 μL mixed inoculum
23 (prepared from mouldy grape leaves to a concentration
24 of 5×10^4 cells/mL). The plates were incubated
25 statically for 24 hours at room temperature and spore
26 growth was visually assessed by microscopy.

27

28 Due to the use of solid medium, the second incubation
29 period in fresh media could not be performed.

30

31

32

1 MIC assay using *Colletotrichum graminicola*

2
3 The different terpene formulations were serially
4 diluted in the 96-well microtitre plate as described
5 for *S. cerevisiae*. *C. graminicola* (300 spores/well)
6 were added to the diluted terpenes and the plates
7 were incubated statically for 48 hours at room
8 temperature. Spore germination and growth were
9 visually assessed by microscopy.

10
11 Once the MIC had been determined for each
12 formulation, the microtitre plates were centrifuged
13 and the spent medium was removed from growth-
14 inhibited wells. The spores were resuspended in
15 fresh medium (100 μ L) and the plates were re-
16 incubated overnight at room temperature. Assessment
17 of growth inhibition was performed as before.

- 1 **Table 17** - MIC and fungicidal MIC values obtained
 2 from initial screening of 31 terpene formulations

	Terpene formulation a	<i>Saccharomyces cerevisiae</i>		Mixed microbes		<i>Colletotrichum graminicola</i>	
		MIC	Cidal MIC	MIC	Cidal MIC	MIC	Cidal MIC
1	Geraniol (G)	500	500	250	NT	63	63
2	Eugenol (E)	500	500	125	NT	125	125
3	Thymol (T)	250	250	63	NT	63	500
4	Citral (C)	250	250	63	NT	125	63
5	L-carvone (L)	250	500	63	NT	125	125
6	GE	1000	2000	125	NT	63	250
7	GT	500	500	250	NT	125	63
8	GC	500	500	125	NT	125	250
9	GL	500	500	125	NT	125	125
10	ET	500	500	125	NT	125	125
11	EC	250	1000	31	NT	125	125
12	EL	500	1000	125	NT	125	125
13	TC	500	500	16	NT	63	63
14	TL	500	1000	63	NT	63	63
15	CL	500	500	≤8	NT	63	63
16	GET	500	500	23	NT	94	94
17	GEC	250	500	94	NT	94	94
18	GEL	500	1000	188	NT	188	188
19	GTC	500	500	47	NT	188	188
20	GTL	500	1000	94	NT	94	94
21	GCL	250	500	94	NT	47	47
22	ETC	125	250	188	NT	94	94
23	ETL	500	1000	≤12	NT	94	94
24	ECL	500	1000	≤12	NT	188	188
25	TCL	500	1000	23	NT	94	375
26	GETC	500	1000	125	NT	250	500
27	ETCL	500	1000	63	NT	125	125
28	GTCL	500	1000	125	NT	250	250
29	GECL	500	1000	≤16	NT	500	500

68

30	GETL	1000	1000	125	NT	500	250
31	GETL	1000	1000	78	NT	625	625
	GET (2:1:2 ratio, w/w/w)	NT	NT	98	NT	78	156
	YP-GET (G:E:T ratio of 2:1:2, w/w) ^b	98	391	98	NT	20	20

NT, not tested; YP-GET, yeast-encapsulated GET
formulation.

^a Terpene combinations were mixed in a 1:1 (w/w) ratio
unless otherwise indicated.

^b MICs calculated by terpene content.

1

2

- 1 **Table 18** - Repeat assay to determine MIC and
 2 fungicidal MIC values

Terpene formulation ^a (by No.)	<i>Saccharomyces cerevisiae</i>		Mixed microbes isolated from mouldy grape leaves ^b		<i>Colletotrichum graminicola</i>	
	MIC	Cidal MIC	MIC	Cidal MIC	MIC	Cidal MIC
T (3)	NT	NT	63	NT	NT	NT
L (5)	NT	NT	250	NT	NT	NT
GE (6)	NT	NT	NT	NT	125	500
EC (11)	125	250	NT	NT	NT	NT
TC (13)	NT	NT	250	NT	63	250
TL (14)	NT	NT	500	NT	250	500
CL (15)	NT	NT	500	NT	125	500
GET (16)	NT	NT	375	NT	188	375
GEC (17)	250	500	NT	NT	NT	NT
GCL (21)	250	500	NT	NT	375	750
ETC (22)	125	250	NT	NT	94	188
ETL (23)	NT	NT	375	NT	188	750
ECL (24)	NT	NT	750	NT	NT	NT
TCL (25)	NT	NT	750	NT	94	375
ETCL (27)	NT	NT	500	NT	63	500
GECL (29)	NT	NT	1000	NT	NT	NT
YP-GET (G:E:T ratio of 2:1:2, w/w) ^c	98	195	NT	NT	39	156

- 3
 4 NT, not tested; YP-GET, yeast-encapsulated GET formulation.
 5 NOTE: Samples were assayed in duplicate. If different values
 6 were obtained between duplicate samples, the higher value has
 7 been presented. No duplicate samples differed by more than one
 8 2-fold dilution.
 9 ^aTerpene combinations were mixed in a 1:1 (w/w) ratio unless
 10 otherwise indicated.

- 1 ^b 1×10^4 cells/mL stock suspension.
2 ^c MICs calculated by terpene content.

3

4 Mixed inoculum

5

6 Using a mixed inoculum presents a number of problems.
7 The variability in spore content between preparations
8 results in poor interassay reproducibility, and
9 growth of contaminating organisms impedes the scoring
10 of spore germination. Unicellular yeast species are
11 particularly problematic in masking spore growth.
12 Although precise data could not be obtained from this
13 assay, an inhibitory effect of terpenes was observed.

14

15 Identification of spores was easier during scoring of
16 the repeat assay than during the initial screening
17 assay as a larger number of spores were used
18 (approximately 50/well versus approximately 10/well).
19 Therefore, data obtained during the repeat assay may
20 provide a more reliable estimate of MIC.

21

22 *Colletotrichum graminicola*

23

24 The generally higher MIC values obtained from the
25 repeat assay compared to the initial screening assay
26 may be due to:

- 27 • use of 1-week-old terpene solutions
28 • use of freshly prepared spores, which had a
29 higher viability than those used in the
30 initial screening assay and may therefore be
31 more difficult to kill.

32

1 **Comparison of terpene formulations as free emulsions**
2 **with the same terpene formulations when encapsulated**
3 **in hollow glucan particles: *Saccharomyces cerevisiae***
4 **MIC assays**

5
6 YPD growth medium (100 μ L) was added to each well of
7 a 96-well microtitre plate and aliquots of different
8 terpene formulations were added to the first row,
9 giving a total volume of 200 μ L in this row. One
10 column was designated as a cell-only control and no
11 terpene was added to these wells. Serial 2-fold
12 dilutions were performed by transferring 100 μ L from
13 one row to the next a total of 7 times. Finally, 100
14 μ L was discarded from the last row in order to ensure
15 that all wells contained the same volume. *S.*

16 *cerevisiae* (5×10^5 cells/mL in YPD growth medium)
17 were then added to each well in 100 μ L aliquots, and
18 the absorbance at 620 nm (A_{620}) was measured for each
19 well using a microtitre plate reader. Microtitre
20 plates were incubated statically overnight at 30°C.

21
22 Following incubation, the A_{620} was measured again and
23 plates were scored for inhibition of growth ($\geq 75\%$).
24 Growth inhibition was visually confirmed by
25 microscopy.

26 For the free terpene emulsions, once the MIC had been
27 determined for each formulation, the microtitre
28 plates were centrifuged and the spent medium was
29 removed from the growth-inhibited wells. The cells
30 were resuspended in fresh medium (100 μ L) and the
31 plates were re-incubated overnight at 30°C.

1 Assessment of growth inhibition was performed as
2 before.

3

4 MIC and fungicidal MIC results are summarised in
5 Table 19.

6

1 **Results**

2 **Table 19** - MIC and fungicidal MIC values obtained
 3 from screening of 31 terpene formulations against
 4 *Saccharomyces cerevisiae*

Terpene formulation ^a (Reference No)	Yeast-encapsulated formulations ^{b, c}		Free terpene emulsions	
	MIC	Cidal MIC	MIC	Cidal MIC
G (1)	111	NT	250	250
E (2)	131	NT	125	250
T (3)	115	NT	125	250
C (4)	118	NT	125	250
L (5)	254	NT	250	500
GE (6)	118	NT	250	500
GT (7)	108	NT	125	250
GC (8)	113	NT	125	250
GL (9)	117	NT	250	500
ET (10)	131	NT	125	250
EC (11)	126	NT	125	250
EL (12)	129	NT	125	250
TC (13)	59	NT	63	63
TL (14)	124	NT	63	125
CL (15)	124	NT	125	125
GET (16)	119	NT	63	125
GEC (17)	119	NT	125	250
GEL (18)	121	NT	125	125
GTC (19)	115	NT	125	125
GTL (20)	119	NT	125	125
GCL (21)	234	NT	125	125
ETC (22)	124	NT	125	125
ETL (23)	123	NT	125	125
ECL (24)	63	NT	63	125
TCL (25)	61	NT	125	500
GETC (26)	61	NT	63	250
ETCL (27)	120	NT	63	125
GTCL (28)	124	NT	125	125

74

GECL (29)	125	NT	125	125
GETL (30)	122	NT	125	250
GECTL (31)	120	NT	125	250
GET (2:1:2 ratio, w/w/w)	125 ^a	NT	125	250
YP-GET (G:E:T ratio of 2:1:2, w/w)	125	NT	125 ^c	250 ^c
YP-ETC (E:T:C ratio of 1:1:1, w/w)	125	NT	125 ^c	250 ^c

NT, not tested; YP-GET, yeast-encapsulated GET formulation; YP-ETC, yeast-encapsulated ETC formulation.

^aTerpene combinations were mixed in a 1:1 (w/w) ratio unless otherwise indicated.

^bYeast-encapsulated formulations unless otherwise indicated.

^cMIC calculated by terpene content.

^dNon-encapsulated emulsion formulation.

1

2 For both the terpene emulsions and yeast-encapsulated
3 terpenes, MICs were typically ≤ 125 ppm, with the most
4 active formulations inhibiting growth at ~ 60 ppm.
5 MIC values obtained for the terpene emulsions were
6 similar to those obtained for their respective yeast-
7 encapsulated formulations. When different values
8 were obtained, they only differed by approximately
9 one 2-fold dilution.

10

11 Many of the free terpene emulsions were fungicidal at
12 the growth inhibitory MIC, with the majority showing
13 fungicidal activity at a 2-fold higher concentration.

14

1 These results demonstrate that terpenes encapsulated
2 in glucan particles are at least as effective at
3 killing fungus as non-encapsulated forms.
4 Additionally the encapsulated compositions used may
5 have had reduced potency due to having been stored
6 for 45 days at 4°C and having a sub-optimal terpene
7 content of ~4% w/w.

8
9 The assay to determine fungicidal activity involves a
10 centrifugation step, which attempts to separate the
11 microbial cells from any residual terpene in the
12 growth medium by producing a pellet of cells at the
13 bottom of the well. This pellet is then resuspended
14 in fresh media and incubated for a second time in the
15 absence of terpene. However, the centrifugation step
16 cannot discriminate between microbial cells and yeast
17 particles, therefore when yeast-encapsulated terpenes
18 are used, the cell pellet will also contain terpene-
19 loaded yeast particles. As a result, both the yeast
20 particles and the microbial cells are then
21 resuspended in the fresh medium.

22
23 This methodology issue is not considered to affect
24 the results obtained in the experiments described
25 above for the following reasons.

- 26
- 27 • In previous experiments, terpene emulsions have
28 been used instead of terpene-loaded yeast particles
29 and fungicidal activity has been clearly shown.
 - 30 • Encapsulated terpenes are released by diffusion,
31 and an equilibrium between the concentration of
32 encapsulated terpenes and the concentration of

1 released terpenes in the surrounding medium is
2 quickly reached. Thus, following centrifugation
3 and resuspension in fresh medium, the concentration
4 of released terpene in the growth medium is likely
5 to be well below that required for growth
6 inhibitory activity.

7 • There was no growth when the contents of the
8 fungicidal MIC well were plated onto solid agar
9 growth medium. When plated onto solid growth
10 medium, diffusion of any residual terpene
11 throughout the large volume of the agar plate
12 results in a local terpene concentration that is
13 too low to cause growth inhibition. The lack of
14 growth from the contents of the fungicidal MIC well
15 must therefore be due to initial fungicidal
16 activity. In contrast, when an MIC was obtained
17 that was lower than the fungicidal MIC and the
18 contents of the MIC well were plated onto solid
19 agar growth medium, growth was observed, indicating
20 a fungistatic effect.

21
22 **Example 16 - Preparation of Encapsulated Terpene**
23 **Compositions for Field Trials**

24
25 The purpose of the following protocol was to
26 encapsulate a terpene composition into hollow glucan
27 particles for subsequent field trials.

28
29 **Materials:**

30 Thymol (supplied by Alpha-Gamma Corporation)
31 Eugenol (supplied by Alpha-Gamma Corporation)
32 Geraniol (supplied by Alpha-Gamma Corporation)

1 1% Tween-80 (supplied by Alpha-Gamma Corporation)
2 Yeast Cell Wall Particles
3 Xanthan gum.
4

5 The yeast cell wall particles were obtained from
6 Biorigin (Sao Paulo, Brazil) under the trade name
7 Nutricell MOS 55, and were manufactured by Açucareira
8 Quatá S.A, Usina Quatá, Quatá - Sao Paulo - Brazil -
9 Zip Code 19780 000. The particles are a spray dried
10 cell wall extract of *S. cerevisiae* and are a free
11 flowing powder of light beige to tan colour.
12

13 **Protocol:** The following protocol was suitable for a 1
14 Kg of particles, but can simply be scaled up for
15 larger production.

- 16 1. Prepare terpene mixture - mix 375 grams of
17 Geraniol + 525 grams Eugenol + 600 grams of
18 Thymol and stir in a glass flask.
- 19 2. Prepare 6.2 L of 1% Tween 80 by mixing 62
20 grams Tween 80 in 6.2 L water in 2 gallon
21 white bucket. Mix to form solution.
- 22 3. Add 6.2 grams Xanthan Gum to Tween solution
23 and stir to dissolve.
- 24 4. Prepare terpene emulsion by mixing 1.5 Kg
25 terpene mixture + 6.2L 1% Tween 80/0.1%
26 Xanthan gum in white bucket using polytron
27 mixer.
- 28 5. Add 1,000 grams of yeast cell wall particles -
29 mix using paint mixer to form uniform
30 suspension.

1 6. Add the terpene emulsion of step 4 to the
2 yeast cell wall particles while mixing to form
3 a thin mayonnaise-like consistency.

4 7. Pour terpene mixture into cans and incubate
5 overnight.

6
7 **Results:** Encapsulated geranoil, eugenol and thymol in
8 hollow glucan particles was obtained as a paste. The
9 paste was easily converted to a dry powder by
10 conventional spray drying techniques. The paste is
11 the "liquid" composition referred to in the following
12 protocols, and the "powder" is the spray dried form.

13
14 **Example 17 - Field trials of Encapsulated Terpene**
15 **Composition on Downy Mildew**

16
17 In grapes, downy mildew is caused by the fungus
18 *Plasmopara viticola*, which infects vineyards
19 worldwide and can cause devastating losses for
20 grape-growers in terms of crop yield and wine
21 quality. The fungus attacks the fruits and all green
22 parts of the vine, causing the leaves to wither and
23 the flowers and berries to rot. The disease
24 manifests as irregular pale yellow or yellow-green
25 spots on the upper surface of leaves, with dense,
26 white-grey, cotton-like fungal growth covering the
27 underside of the leaf lesions. Berries may also be
28 covered with the downy growth and, depending on the
29 time of infection, may turn brown and soft or may not
30 soften at all. Downy mildew is spread through the
31 dispersal of spores by the wind and rain, and
32 requires wet conditions for infection. It is

1 particularly problematic in environments with high
2 humidity. Preventative measures are recommended for
3 management of the disease, with early applications of
4 fungicides followed by repeat applications at
5 appropriate intervals. Resistance has arisen to some
6 treatments, and although the development of
7 resistance can be minimised by rotating the use of
8 different fungicides, it remains a problem.

9

10 The purpose of this trial was to investigate the
11 efficacy of the encapsulated terpene formulation of
12 Example 16 (YGP-GET) supplied as a liquid or powder
13 (spray dried) formulation, for the prevention of
14 downy mildew in grapes.

15

16 Four adjacent blocks, each covering 0.1 ha, were
17 identified on site 20 in the Kir-Yianni vineyard.

18

19 Kir-Yianni is a 35 ha vineyard at an elevation of 300
20 m. It is bordered by a mixed oak forest on the north
21 and west, and overlooks orchards and vineyards to the
22 south and east.

23

24 All four blocks had been treated with multiple
25 products prior to application of the terpene
26 formulation. On 26 June 2004, two of the four blocks
27 were sprayed with the terpene powder formulation at a
28 dose of either 0.5 g/L or 2 g/L (see schematic
29 illustration in Figure 21). A third block was
30 treated with conventional Bordeaux mix plus wettable
31 sulphur, and the remaining block was left untreated.

1 The vines in each block were monitored for signs of
2 downy mildew over the following week.

3

4 Four further adjacent blocks, each covering 0.1 ha,
5 were identified on site 18 in the Kir-Yianni
6 vineyard. All four blocks had been treated with
7 multiple products prior to application of the terpene
8 formulation. On 26 June 2004, two of the four blocks
9 were sprayed with the terpene liquid formulation at a
10 dose of either 1 g/L or 4 g/L (Figure 21) (note: 1 g
11 of the terpene liquid formulation has a volume of 1
12 ml). Of the remaining two blocks, one was left
13 untreated and one was sprayed with Mikal®, a
14 conventional treatment for downy mildew, on 28 June
15 2004. The vines in each block were monitored for
16 signs of downy mildew over the following week.

17

18 For both sites, the terpene product was applied at a
19 rate of 1200 L/ha.

20

21 The following growth stages of the grapes were
22 recorded:

- 23 - bud break, 26 March 2004
- 24 - bloom, 1 June 2004
- 25 - veraison, 6 August 2004

26

27 The study applications took place pre-veraison.

28

29 The 2004 growing season was exceptionally late and
30 was wet throughout. Disease pressure from downy
31 mildew was extremely high, *botrytis* levels were
32 elevated, and powdery mildew pressure was moderate.

1 Both the powder and liquid YGP-GET formulations were
2 stored at room temperature. No special storage
3 conditions were used.

4

5 **Details of Comparator Products**

6

7 Powder formulation trial: Bordeaux mix, manufactured
8 by Manica Spa, Italy, packed in Greece by Moscholios
9 Chemicals SA; wettable sulphur.

10

11 Liquid formulation trial: Mikal® (fosetyl-al 50%,
12 folpet 25%), manufactured by Bayer CropScience,
13 distributed in Greece by Bayer Hellas SA.

14 The comparator products were applied as follows:
15 One application before bud-break at a dosage of
16 15 g/L followed by two more applications per year at
17 a dosage of 6.5 g/L. A spraying rate of 1000 L/ha
18 was used for all three applications.

19

20 Powder formulation trial: Bordeaux mix (2 g/L) and
21 wettable sulphur (2.2 g/L) were applied on 26 June
22 2004.

23

24 Liquid formulation trial: Mikal (3.2 g/L) was
25 applied on 28 June 2004.

26

27 Vines were visually examined for symptoms of downy
28 mildew. Onset of the disease was marked by an
29 average of two oily spots per leaf. Treatments that
30 prevented the appearance of further spots were
31 considered to provide effective protection against
32 downy mildew.

1 **Results**

2 **YGP-GET powder formulation (spray dried)**

3 The conventional treatment of Bordeaux mixture
4 provided good protection against downy mildew. Mild
5 symptoms of downy mildew were observed in the control
6 vines. The 0.5 g/L terpene product concentration did
7 not provide protection, and the 2 g/L terpene product
8 concentration provided only slightly better
9 protection than the control. Note: the disease
10 pressure at this site was very low because of the
11 recent pesticide treatment.

12
13 Difficulties were encountered in dissolving the
14 powder formulation as it was very fine, resulting in
15 dispersion in the air. This may have adversely
16 affected the efficacy of the product.

17

18 **YGP-GET liquid formulation**

19 When administered at a dose of 4 g/L, the terpene
20 product provided excellent protection against downy
21 mildew on exposed canopy. No protection was provided
22 by the 1 g/L dosage. Serious symptoms of downy
23 mildew were observed in the control block.

24

25 The liquid formulation was easy to use and had a
26 pleasant odour.

27

28 **Discussion**

29 Downy mildew can cause devastating losses for
30 grape-growers because of its effects on crop yield
31 and wine quality. Management of the disease focuses
32 on prevention because, once established, the

1 infection can quickly spread. At the site sprayed
2 with the powder formulation, YGP-GET did not exhibit
3 efficacy at the lower dosage (0.5 g/L), and the dose
4 of 2 g/L was less effective than the conventional
5 treatment. At this site, the recent pesticide
6 applications resulted in low disease pressure, which
7 may have limited the apparent efficacy of the terpene
8 treatment. However, it was considered that a dosage
9 of less than 2 g/L of the terpene product was
10 inadequate.

11

12 At the site sprayed with the liquid formulation,
13 excellent protection of exposed canopy was provided
14 by the higher dose level of 4 g/L. Excessive
15 vegetative growth at this site resulted in more
16 effective treatment of the outer, younger branches
17 compared with the older growth in the inner canopy.
18 Complete foliar coverage by the terpene product is
19 useful, as the treatment is not systemic. It is
20 estimated that an approximately 30% increase over the
21 volume used for conventional systemic treatments
22 would achieve good coverage using the terpene
23 treatment.

24

25 **Conclusions:**

26 Foliar application of YGP-GET liquid formulation was
27 highly effective at controlling downy mildew at a
28 concentration of 4 g/L. The lower concentrations of
29 0.5 g/L powder and 1 g/L liquid were not effective.

30

31

1 **Example 18 - Field trials of Encapsulated Terpene**
2 **Composition on Powdery Mildew**

3
4 Powdery mildew of grapes is caused by the fungus
5 *Uncinula necator*, and causes reductions in vine
6 growth, fruit quality and winter hardiness of vines.
7 In wine grapes, an infection level of only 3% of
8 berries can affect wine quality. The disease is
9 characterised by small white-grey patches of fungal
10 growth that enlarge into a powdery, white coating on
11 the leaves. The fungal growth can also occur on the
12 berries, which may split. In contrast to downy
13 mildew, which requires warm wet conditions, powdery
14 mildew can be a problem in drier growing seasons, as
15 it favours shaded areas with humid but not rainy
16 weather conditions. Preventative measures are
17 recommended for management of powdery mildew, with
18 early applications of fungicides followed by repeat
19 applications at appropriate intervals.

20
21 This study aimed to investigate the efficacy of
22 application of the YGP-GET composition for the
23 prevention of powdery mildew in grapes.

24
25 Three adjacent blocks, each covering 0.1 ha, were
26 identified on site 18 in the Kir-Yianni vineyard. On
27 19 July 2004, one of the three blocks was sprayed
28 with the YGP-GET liquid formulation at a dose of
29 2 ml/L and one was left untreated. The remaining
30 block was sprayed with the conventional treatment of
31 Equesion (2.5 g/L), Alliete (0.9 g/L) and Punch
32 (0.075 mL/L) (see Fig.22). The vines in each block

1 were monitored for signs of powdery mildew over the
2 following week.

3
4 Three further adjacent blocks, each covering 0.1 ha,
5 were identified on site 20 in the Kir-Yianni
6 vineyard. On 20 July 2004, one of the three blocks
7 was sprayed with the YGP-GET liquid formulation at a
8 dose of 2 mL/L and the two remaining blocks were left
9 untreated (see Fig. 22). The vines in each block
10 were monitored for signs of powdery mildew over the
11 following week.

12
13 At both sites, the blocks had previously been treated
14 with multiple products, including a prior application
15 of terpene product.

16
17 All terpene treatments were applied at a rate of 1200
18 L/ha to ensure complete coverage.

19
20 The following growth stages of the grapes were
21 recorded

- 22 - bud break, 26 March 2004
- 23 - bloom, 1 June 2004
- 24 - veraison, 6 August 2004

25
26 The study applications took place pre-veraison.

27
28 The 2004 growing season was exceptionally late and
29 was wet throughout. Disease pressure from downy
30 mildew was extremely high, botrytis levels were
31 elevated, and powdery mildew pressure was moderate.

32

1 **Details of Comparator Products**

2 No comparator product was used at site 20. The
3 comparator treatment used at site 18 is detailed
4 below.

5
6 Punch® (flusilazole 40%), DuPont.

7 On 19 July 2004, Punch was applied at a dose of 0.075
8 ml/L as a preventative treatment for powdery mildew
9 according to the manufacturer's instructions.

10

11 **Details of Additional Products**

12 No additional products were used at site 20. The
13 additional products used at site 18 are detailed
14 below.

15

16 Equesion system (famoxadone 22.5% plus cymoxanil 30%)
17 Alliete (fosetyl-al 80%)

18

19 On 19 July 2004, Equesion (2.5 g/L) and Alliete (0.9
20 g/L) were applied as preventative treatments for
21 downy mildew. The dose was determined according to
22 the manufacturer's instructions.

23

24 The comparator and additional products represent
25 conventional treatments in the integrated pest
26 management schedule.

27

28 Vines were visually examined for symptoms of powdery
29 mildew.

30

31

32

1 **Results:**

2 Site 18

3 Approximately 20% of the peduncles and stems in the
4 control block were black, indicating moderate
5 infection from powdery mildew. In both the
6 conventional treatment block and the terpene-treated
7 block, all stems and bunches were green, indicating
8 that adequate protection had been provided.

9

10 Site 20

11 No evidence of powdery mildew infection was observed
12 in any of the blocks.

13

14 **Additional observations**

15 At the end of the growing season, the blocks at sites
16 18 and 20 generally showed less stress due to disease
17 than the rest of the vineyard.

18

19 Powdery mildew infections cause considerable losses
20 to growers through reductions in vine growth, fruit
21 quality and winter hardiness of vines. Furthermore,
22 wine quality can be affected by an infection level of
23 as little as 3% of berries. Management of the
24 disease focuses on prevention because, once
25 established, the infection can quickly spread. In
26 this study, the application of terpene product YGP-
27 GET at site 18 effectively prevented powdery mildew
28 infection, and the level of control exhibited by the
29 terpene product was comparable to that provided by
30 the conventional treatment. The results from site 20
31 are inconclusive, however, due to the lack of powdery
32 mildew infection. This lack of infection is likely

1 to be due to the extensive application of pesticides
2 prior to the study, which resulted in low disease
3 pressure.

4
5 The lower level of stress due to disease at sites 18
6 and 20 suggests that the earlier terpene treatment
7 applied at these sites may have been beneficial in
8 control of infection in the long term.

9
10 **Conclusions:**

11 YGP-GET effectively prevented powdery mildew
12 infection, with a comparable level of control to that
13 provided by the conventional treatment.

14
15 **Example 18 - Further Field Trials of Encapsulated**
16 **Terpene Composition on Powdery Mildew**

17
18 The study aimed to further investigate the efficacy
19 of YGP-GET for the treatment of powdery mildew in
20 Grimson Seedless table grapes.

21
22 A 0.1 ha plot on the Tsigaras vineyard (approximately
23 80 km south of the Kir-Yianni vineyard) was
24 inadvertently left untreated during an application of
25 Cisteine on 1 July 2004. The vines in this plot
26 subsequently showed severe symptoms of powdery mildew
27 on the leaves, stems and grapes. On 12 July 2004,
28 the untreated plot was sprayed with 3 ml/L liquid
29 YGP-GET formulation at a rate of 1200 l/ha, and the
30 rest of the vineyard was sprayed with the comparator
31 product Rogana. The vines were assessed for symptoms
32 of powdery mildew after 24 hours.

1 Vines were trained in a high lyre trellis system.

2

3 **Details of Comparator Product**

4 Rogana (fenbuconazol 5%, binocap 16%), manufactured
5 by BASF (BASF Agro Hellas S.A., Athens, Greece)

6 On 12 July 2004, Rogana was applied to the Tsigaras
7 vineyard as a treatment for powdery mildew. The dose
8 was determined according to the manufacturer's
9 instructions.

10

11 Vines were visually examined for symptoms of powdery
12 mildew.

13

14 **Results**

15 Severe symptoms of powdery mildew were evident prior
16 to application of YGP-GET. Only 24 hours after YGP-
17 GET application, the white bloom of the powdery
18 mildew turned black, indicating effective antifungal
19 activity. As the disease was effectively halted at
20 this time, no further treatments were applied. YGP-
21 GET showed comparable efficacy to the conventional
22 treatment.

23

24 **Discussion:**

25 In this study, an established powdery mildew
26 infection was treated quickly and effectively using
27 YGP-GET. Only 24 hours after application, the
28 previously severe powdery mildew infection was halted
29 by application of the terpene product, with
30 comparable efficacy to the conventional treatment.

31

1 The preliminary data obtained from this study suggest
2 that YGP-GET may be efficacious in treating
3 established fungal infections in addition to showing
4 preventative ability.

5

6 **Example 19 - Further Field trials of Encapsulated**
7 **Terpene Composition on Powdery Mildew**

8

9 **Background and Rationale**

10 In the current trial, the use of YGP-GET was
11 investigated as part of a Tasmanian vineyard's
12 (Frogmore Creek Vineyard, Hathaway Trading Pty Ltd,
13 Box 187, Richmond TAS 7025, Australia) experimental
14 programme to control powdery mildew using organic
15 products. The aim of this study was to investigate
16 the short-term efficacy of the application of YGP-GET
17 in the organic control of powdery mildew in
18 Chardonnay grapevines.

19

20 In this trial grapevines (Chardonnay variety) were
21 either treated with the terpene product YGP-GET or
22 left untreated (control) on 7 February 2005. Although
23 suppressed by previous organic treatments, the pre-
24 trial severity of powdery mildew was at a level
25 considered unacceptable commercially and was
26 equivalent in the 6 active-treatment plots and 6
27 control plots. The crop stage was approximately E-L
28 33-34 (pre-veraison).

29

30 YGP-GET (4 mL/L) (liquid formulation) was sprayed
31 onto 6 Chardonnay plots, which had been treated
32 previously with milk. Six Chardonnay plots served as

1 untreated controls, but they had been treated
2 previously with oil/whey. The number of vines per
3 plot was typically 7.

4

5 Details of the composition of the YGP-GET used in
6 this protocol are given in Table 20.

7

8 **Table 20** - Formulation of Batch Used in Present Study

9

Raw material mix details	Weight in lbs	% by Weight
Geraniol	323.52	6.88
Eugenol	161.76	3.44
Thymol	323.52	6.88
Yeast particles	722.13	15.35
Xanthan	3.17	0.07
Polysorbate	3.17	0.07
Water	3166.62	67.32
TOTAL	4703.89	100.00

10

11 The severity of powdery mildew was assessed 3 days
12 before terpene treatment and again 3 days post-
13 treatment. In each plot, 20 grape bunches were
14 selected at random (10 bunches per panel side), and
15 disease severity was estimated as the percentage area
16 of the bunches covered with active mildew colonies.
17 No further assessment was possible because the grower
18 subsequently sprayed the entire trial area with

1 sulphur and a vegetable oil-based spraying adjuvant
2 (Synertrol Horti Oil).

3

4 **Number/area of plants to be treated**

5 Test product: YGP-GET (4 mL/L) to be applied to 6
6 Chardonnay plots (total of approximately 42 vines),
7 which had been treated previously with milk.

8

9 Control: No treatment was applied to 6 Chardonnay
10 plots (total of approximately 42 vines) to be used as
11 controls, but they had been treated previously with
12 oil/whey.

13

14 **Cultivation methods**

15 *Vitis vinifera* (Chardonnay) vines in Block B2:
16 vertical shoot positioning with arched canes.

17

18 **Cultivation arrangement**

19 Spacing: Distance of 2.5 m between rows and 1.25 m
20 between vines (within row), with 3,200 vines per
21 hectare. Row orientation was north to south.

22

23 **Canopy density**

24 The point-quadrat method was used to characterise the
25 pre-trial canopy density of the Chardonnay vines
26 (Table 21). Measurements were taken on 13 January
27 2005 by selecting representative sections of the
28 canopy within the Chardonnay plots that previously
29 had been either treated with sulphur or left
30 untreated. Ten measurements were taken in each of the
31 6 plots of each prior treatment (i.e. a total of 60
32 measurements for the sulphur-treated plots and 60

1 measurements for the untreated control plots). In
 2 addition, the length and number of nodes on 3 upright
 3 shoots (per plot) were measured.

4

5 **Table 21** - Pre-trial canopy density of the Chardonnay
 6 vines

Prior treatment	Gaps (%)	Leaf layer number (LLN)	Interior leaves (%)	Interior clusters (%)	Mean number of nodes	Mean shoot length (cm)
Untreated	12	1.5	22	26	21	110
Sulphur	5	2.0	27	40	21	104
Optimum values	20-40%	≤1.0-1.5	<10%	<40%	NA	NA

7

8 NA, not applicable.

9

10 **General condition**

11 Previous treatment of these plots with experimental
 12 materials suppressed powdery mildew in comparison to
 13 the untreated control. However, the level of powdery
 14 mildew was considered commercially unacceptable,
 15 although equivalent in both the milk- and oil/whey-
 16 treated plots.

17

18 **Application method, dose and regimen**

19 YGP-GET treatment (4 mL/L) was applied on 7 February
 20 2005 with a hand gun connected to a hose reel and
 21 pump mounted on the flat tray of a utility vehicle.
 22 The spray was propelled with a pump pressure of 1500-

1 1600 kPa (200-230 psi), delivering approximately 63
2 mL/second. The standard spray volume for
3 conventional treatments (approximately 900 L/ha) was
4 used.

5
6 The severity of powdery mildew, estimated as the area
7 (%) of the grape bunches covered with active mildew
8 colonies, was assessed for 20 bunches selected at
9 random within each plot (10 bunches per panel side).
10 Disease severity was assessed on 4 February 2005,
11 3 days before application of the YGP-GET treatment,
12 and again on 10 February 2005, 3 days after terpene
13 application.

14
15 Data were transformed using arcsin transformation to
16 obtain mean separations.

17

18 **Results**

19 Prior to treatment, the mean severity of powdery
20 mildew on Chardonnay grape bunches in the 6 plots to
21 be treated with terpene (20.4%) was similar to that
22 in the 6 control plots (23.2%; Table 22). Statistical
23 analysis based on arcsin transformation of these data
24 found that there was no significant difference in
25 disease severity before treatment (Table 23).

26

27 Three days after treatment, however, the mean
28 severity of powdery mildew was 23.8% on the YGP-GET -
29 treated bunches versus 37.8% on the controls (Table
30 22). Arcsin transformation of these data showed a
31 statistically significant difference in favour of the
32 terpene-treated grape bunches, which had a smaller

1 area covered with active mildew colonies ($p = 0.058$;
2 Table 23).

3

4 **Table 22.** Mean severity of powdery mildew (%) on
5 Chardonnay bunches before and after treatment with
6 YGP-GET

Treatment applied on 7 Feb 2005	Mean severity	
	On 4 Feb 2005	On 10 Feb 2005
YGP-GET	20.4	23.8
None	23.2	37.8

7

8 **Table 23.** Statistical separation of treatments
9 following arcsin transformation of data

Treatment applied on 7 Feb 2005	Mean severity (SEM)	
	On 4 Feb 2005	On 10 Feb 2005
YGP-GET	0.2063 (0.03857)	0.2411 (0.04303)
None	0.2401 (0.08534)	0.3954 (0.07852)
	$t = 0.36$ $df = 10$ $p = 0.726$ Two-sided test: difference not significant	$t = 1.72$ $df = 10$ $p = 0.058$ One-sided test: untreated > treated

10

11

1 **Discussion:**

2 Infection of grapevines with powdery mildew can cause
3 considerable losses to growers through detrimental
4 effects on vine growth and hardiness, as well as on
5 the quality of the fruit and wine. In organically
6 managed vineyards, growers are searching for
7 alternatives to treatments such as elemental sulphur.

8

9 This study investigated the efficacy of encapsulated
10 terpene formulations (4 mL/L) as a liquid formulation
11 in controlling powdery mildew in an organic vineyard
12 in Tasmania, Australia. While other experimental
13 treatments had been used as little as 3 weeks before
14 terpene application, the level of powdery mildew
15 infection was still considered commercially
16 unacceptable. Three days after treatment of
17 Chardonnay vines with YGP-GET, the severity of
18 powdery mildew on treated grapes was significantly
19 less than that on untreated controls. While the
20 severity of infection in untreated controls worsened
21 during the 6 days between pre- and post-treatment
22 assessments, it remained steady in treated vines.
23 Therefore, YGP-GET appeared to have slowed the rate
24 of disease increase on grape bunches that had well-
25 established colonies of sporulating powdery mildew
26 before treatment. Presumably, colony expansion was
27 inhibited, although existing colonies continued to
28 sporulate to some degree. More long-term assessment
29 of efficacy was not possible because the grower
30 subsequently sprayed the entire trial area with
31 sulphur.

32

1 These encouraging results demonstrate the efficacy of
2 YGP-GET in controlling powdery mildew in grapevines.

3

4 **Example 20 - Field Trials of Encapsulated Terpene**
5 **Composition on Botrytis**

6

7 Botrytis bunch rot of grapes is caused by *Botrytis*
8 *cinerea*, a common fungus that can cause serious
9 losses in fruit yield. Berries are the predominant
10 site of infection, although the disease can also
11 affect blossom and leaves. Initially, infected
12 berries appear soft and watery, and may become
13 covered with grey fungal growth in conditions of high
14 humidity and moisture. Over time, infected berries
15 shrivel and drop. Botrytis favours humid conditions
16 with poor air circulation, and split or damaged
17 berries are particularly susceptible to the spread of
18 infection. Management strategies for botrytis
19 include promotion of good air circulation, prevention
20 of wounding and application of fungicides at
21 appropriate times during the growing season.

22

23 The aim of this study was to investigate the efficacy
24 of YGP-GET in the treatment of botrytis infection in
25 grapes.

26

27 The emergence of botrytis in the Kir-Yianni vineyard
28 in mid October 2004 (3 weeks after an application of
29 Teldor® could not be treated with conventional
30 agrochemicals because the associated re-entry time
31 restrictions would prevent the planned harvest. Two
32 adjacent 0.1 ha plots were therefore identified on

1 site 7 of the vineyard, and, on 12 October 2004, one
2 of these plots was treated with 4 mL/L YGP-GET liquid
3 formulation and the other was left untreated (see
4 Fig. 23). The crop was harvested 3 days later, and
5 the proportion of infected berries was determined for
6 each plot (percentage weight of total yield).
7 Uninfected berries from both the treated and
8 untreated plots were then mixed in the fermentation
9 tank.

10

11 Site 7 had been treated with multiple products prior
12 to the application of the terpene formulation but
13 still showed botrytis infection.

14

15 Vines were given a single application of 4 mL/L YGP-
16 GET liquid formulation at a rate of 1200 l/ha.

17

18 The following growth stages of the grapes were
19 recorded:

- 20 - bud break, 26 March 2004
- 21 - bloom, 1 June 2004
- 22 - veraison, 6 August 2004
- 23 - harvest, 15 October 2004

24

25 The study applications took place 3 days before
26 harvest.

27

28 The 2004 growing season was exceptionally late and
29 was wet throughout. Disease pressure from downy
30 mildew was extremely high, powdery mildew pressure
31 was moderate and botrytis levels were elevated.

32

1 YGP-GET was applied at this time to assess its
2 potential efficacy against a botrytis infection that
3 could not otherwise have been treated because of
4 pesticide time restrictions prior to harvest.

5
6 Visual assessment of the site prior to terpene
7 product application revealed evidence of botrytis
8 infection. After harvest, the berries were displayed
9 on a conveyor belt and infected berries were manually
10 separated from uninfected berries prior to crushing.
11 The proportion of infected berries was calculated as
12 a percentage of the total yield (by weight) for each
13 plot.

14 15 **Results**

16 Visual assessment of the site prior to YGP-GET
17 application revealed evidence of botrytis infection.
18 Following harvest (3 days after YGP-GET application),
19 the proportions of infected berries were 13% and 23%
20 in the treated and untreated plots, respectively.
21 The tested areas were not sufficient to assess
22 statistical significance; however, YGP-GET treatment
23 clearly slowed the progression of the disease.

24
25 Fermentation was not affected by the mixing of
26 uninfected berries from the untreated and terpene-
27 treated plots.

28 29 **Discussion**

30 Conventional treatments for botrytis must be halted 3
31 weeks before harvest, leaving time for considerable
32 damage to crop yield and quality to occur. The

1 development of a treatment that could be used until
2 harvest, or that could be continued closer to harvest
3 than the existing products, could result in
4 significant improvements in crop yield and wine
5 quality, and would be of considerable benefit to
6 growers. In this study, treatment with the terpene
7 product YGP-GET visibly slowed progression of an
8 established botrytis infection only 3 days prior to
9 harvest, resulting in a lower proportion of infected
10 berries in the terpene-treated plot than in the
11 untreated plot. Furthermore, despite the use of YGP-
12 GET close to harvest, fermentation was unaffected by
13 the combination of treated and untreated grapes.

14

15 These results suggest that YGP-GET is efficacious in
16 reducing the impact of established botrytis
17 infections and can be used near to harvest without
18 detrimental effects on subsequent fermentation.

19

20 **Example 21 - Evaluation of Encapsulated Terpenes for**
21 **the Treatment of established Downy Mildew and**
22 **subsequent evaluation of grape quality**

23

24 A trial of YGP-GET was carried out on 25/08/04
25 applying the composition at a rate of 1000 g per 250
26 liters.

27

28 A vineyard of Cabernet Sauvignon which was 100%
29 infected and suffering substantial leaf loss due to
30 Downy Mildew was sprayed. Any remaining leaves were
31 infected with spots of Downy Mildew as evidenced by
32 the yellow spot on top of the leaf and the fuzzy

1 growth on the leaf bottom; the classical indication
2 of Downy Mildew. Many of the leaves were almost
3 entirely yellow indicating substantial infection.
4 This leaf loss and the infection in general delays
5 the maturity of the grapes and in many cases the
6 grapes never fully ripen for winemaking purposes.

7
8 Observation of totally unripened (i.e. hard dark
9 green berries ~ 1 cm diameter and oval in shape)
10 bunches occasionally in the vines indicated that the
11 vines were likely infected before veraison, and
12 likely at bloom or before. No early copper (Bordeaux
13 or basic Copper sulfate) application has been used.
14 This vineyard was heavily infected in the previous
15 harvest to the point that no crop was produced from
16 the Cabernet Sauvignon. Leaf loss last year was 100%
17 despite Potassium Bi-carbonate treatment in an
18 attempt to contact kill the Downy Mildew, followed by
19 Stilbourin application for longer term systemic
20 protection.

21

22 On 19/09/04 the grapes treated in this trial were
23 picked and crushed and the following observations
24 were made on the must (Table 24):

25

26 **Table 24**

	Control	Treated	Desirable
pH	3.28	3.30	3.3-3.5
TA	0.92	0.85	0.7-0.75
Brix	17.4	18.7	20-22

27

1 These results indicate the grapes from the treated
2 vines are riper than those of the untreated vines.
3 Observation of the grapes themselves indicated that
4 the untreated grapes were, on average, lighter in
5 color, some with a transparent pinkish/purple/green
6 tint, indicative of grapes just past veraison,
7 whereas the treated grapes were dark purple on
8 average and opaque, typical of fully or nearly fully
9 ripened grapes.

10

11 Tasting of these grapes revealed the treated grapes
12 to have a fuller fruitier taste typical of ripe
13 Cabernet Sauvignon, whereas the untreated grapes did
14 not have the full fruity taste. The untreated grapes
15 had a green apple sour taste indicating probable a
16 high malic/tartaric ratio unsuitable for good
17 winemaking.

18

19 These grapes were crushed and destemmed in
20 preparation for producing a wine from these grapes to
21 demonstrate the difference in these grapes and to
22 demonstrate the suitability of the treated grapes for
23 winemaking. The grape grower was concerned that this
24 treatment would affect the flavor of the wine,
25 although at my suggestion he tasted treated grapes
26 the day after application of YGP-GET and found no
27 lingering taste or aroma.

28

29 The difference in the treated and untreated grapes is
30 further demonstrated in the color of the must. The
31 juice of the untreated grapes was light
32 greenish/uncolored (somewhat like a white wine must)

1 whereas the must from the treated grapes was a
2 pinkish color typical of ripe Cabernet Sauvignon
3 grapes immediately after crushing.

4 These results indicate that YGP-GET is efficacious in
5 late summer vineyard treatment by killing and
6 stopping Downy Mildew re-infection, in at least the
7 short term.

8
9 Further research into the long term efficacy of the
10 YGP-GET in controlling downy mildew would be useful,
11 but the results presented show that YGP-GET is a
12 useful treatment.

13
14 Late onset Downy Mildew can completely ruin a crop
15 and there are currently no effective treatments which
16 can be applied shortly before harvest and that retain
17 their ability to provide protection. The great
18 strength of YGP-GET is the ability to provide a quick
19 kill and maintain this efficacy over a longer time
20 than other contact fungicides.

21
22 There are a number of anti-fungals in this market
23 which have an established track record against Downy
24 Mildew, but all need some time after application
25 before the crop can be harvested. Some treatments
26 (like sulfur containing products) cannot be used if
27 the temperature rises above 85°F. Phytotoxicity of
28 copper containing fungicides is also significant
29 depending on the variety of grape. Contact
30 fungicides do not have a long term effect so a second
31 application of a longer active fungicide is often

1 needed, but may be restricted by relevant regulation
2 (e.g. PHI or REI).

3

4 Many conventional treatments for Downy Mildew have a
5 restricted reentry (REI and or PHI) which means the
6 grower cannot apply the treatment in fear that he
7 will apply something like Mancozeb, which has a PHI
8 of 66 days; the grower would then be unable to
9 harvest his grapes at peak maturity.

10

11 Downy Mildew is implicated as the primary cause of
12 the many poor wines being produced east of the
13 Mississippi. YGP-GET could allow affected grapes to
14 ripen properly and be picked at peak maturity in this
15 rapidly growing industry.

16

17 Advantageously YGP-GET should be eligible for
18 approval by the various "organic" committees (many
19 self-appointed) that this product is suitable for use
20 on grapes grown under "organic" guidelines. This
21 opens another niche in a rapidly growing market
22 segment in the US and worldwide.

23

24 **Example 22 - In vitro assessment of the fungicidal**
25 **properties of encapsulated and non-encapsulated**
26 **terpenes**

27

28 Further tests were conducted to assess the 31 non-
29 encapsulated terpene preparations set out in Example
30 15 and preparations 16 and 22 encapsulated in glucan
31 particles.

32

1 To conduct these assays, 20,000 spores were placed in
2 1/3 strength potato dextrose broth (PDB) and
3 sufficient quantities of selected terpene
4 formulations were added to give concentrations
5 ranging from 10 to 1000 ppm. These test materials
6 were placed in separate sterile capped Eppendorf
7 tubes with *Botrytis cinerea* (B.c.) spores, incubated
8 for 24 hr, then the spores were recovered by
9 centrifugation, and the terpene solutions were
10 discarded. The spores/biomass were rinsed with
11 sterile water, centrifuged again and then taken back
12 up in 300 µl of 1/3 strength PDB and transferred to
13 96 well plates. The optical density of the surviving
14 spores growing into mycelia was measured over time.
15 Fungicidal activity is defined as total killing of
16 20,000 spores after 24 hours terpene exposure, as
17 evidence by the absence of mycelial growth.

18

19 The results suggest that certain formulations were
20 not fungicidal at a statistically significant level
21 under the present test conditions (results not
22 shown). These were:

23

24 1, 2, 4, 5, 6, 8, 9, 11, 12, 14, 15, 17, 18, 19, 20,
25 21, 23, 24, 25, 27, 28, 29, 30. Refer to Example 15
26 (Table 17) for details of the compositions.

27

28 The minimum inhibitory concentration for the most
29 effective compounds is set out in Table 26.

30

31

32

1 **Table 25**

Material	Minimum inhibitory concentration (ppm)	Material	Minimum inhibitory concentration (ppm)
3	<1000; >750	7	<1000; >750
10	<1000; >500*	13	<1000; >750
16	<1000; >750	22	<750; >500
26	<1000; >750	31	<1000; >750

2 *In different tests, the lowest concentration that
3 gave no growth was either 500 or 750 ppm.

4

5 **Comparative testing of compounds in water and**
6 **encapsulated in hollow glucan particles.**

7

8 Samples of formulations 16 (geraniol, eugenol and
9 thymol) and 22 (eugenol, thymol and citral)
10 encapsulated in hollow glucan particles were prepared
11 in accordance with techniques previously described.
12 The fungicidal properties were then assessed for
13 encapsulated and non-encapsulated formulations using
14 the protocol previously described for the non-
15 encapsulated formulations.

16

17 The results were quite different with encapsulated
18 terpene formulations as compared with the terpenes
19 suspended in water, as shown in Fig 24.

20

21 The minimum effective concentration is shown below in
22 Table 26.

23

1 **Table 26**

Material	MIC in suspension	MIC in yeast particles
16	<1000, >750	<100, >250
22	<750, >500	<500, >250

2

3 Thus, the results with materials 16 and 22 are quite
4 different when in aqueous suspension and when tested
5 encapsulated in glucan particles. (Note: as mentioned
6 later, there was some variability in the results with
7 terpenes suspended in water, the experiment noted
8 above is an example of this). The MIC values are
9 composites from several trials. Importantly, the
10 results with encapsulated terpene formulations do not
11 suffer from the problems of variability associated
12 with aqueous terpene suspensions. There have been
13 five separate tests of terpenes suspended in water
14 and three with the YPs.

15

16 Encapsulated terpene formulations are readily
17 miscible with water and provide a slow release
18 terpene formulation into the aqueous medium. This
19 results in a longer exposure time of the spores to
20 the terpenes.

21

22 Problems monitoring the non-encapsulated terpene
23 formulations in suspension in the test media were
24 encountered which may have affected the results in
25 this regard.

1 **Claims**

2

3 1. A composition comprising a hollow glucan particle or a
4 cell wall particle encapsulating a terpene component.

5

6

7 2. A composition according to claim 1 wherein the
8 hollow glucan particle or cell wall particle is
9 a fungal cell wall.

10

11 3. A composition according to claim 2 wherein the
12 hollow glucan particle or cell wall particle is
13 a yeast cell wall.

14

15 4. A composition according to claim 3 wherein the
16 yeast cell wall is derived from a Baker's yeast
17 cell.

18

19 5. A composition according to any preceding claim
20 wherein the hollow glucan particle or cell wall
21 particle is an insoluble waste product from a
22 yeast extract manufacturing process.

23

24 6. A composition according to any one of claims
25 any preceding claim wherein the hollow glucan
26 particle or cell wall particle has been alkali
27 extracted.

28

29 7. A composition according to any preceding claim
30 wherein the hollow glucan particle or cell wall
31 particle has been acid extracted.

32

- 1 8. A composition according to any preceding claim
2 wherein the hollow glucan particle or cell wall
3 particle has been organic solvent extracted.
4
- 5 9. A composition according to any preceding claim
6 wherein the hollow glucan particle or cell wall
7 particle has a lipid content of 1% or greater.
8
- 9 10. A composition according to claim 9 wherein the
10 lipid content of the hollow glucan particle or
11 cell wall particle is 5% w/w or greater.
12
- 13 11. A composition according to claim 10 wherein the
14 lipid content is 10% w/w or greater.
15
- 16 12. A composition according to any preceding claim
17 wherein the terpene component comprises one or
18 more of the terpenes selected from the group
19 consisting of citral, pinene, nerol, b-ionone,
20 geraniol, carvacrol, eugenol, carvone (for
21 example L-carvone), terpeniol, anethole,
22 camphor, menthol, thymol, limonene, nerolidol,
23 farnesol, phytol, carotene (vitamin A₁),
24 squalene, thymol, tocotrienol, perillyl
25 alcohol, borneol, myrcene, simene, carene,
26 terpenene, linalool or a mixture thereof.
27
- 28 13. A composition according to any preceding claim
29 wherein the terpene component comprises a
30 terpene having have the general structure
31 C₁₀H₁₆.
32

- 1 14. A composition according to any preceding claim
2 wherein the terpene component comprises one or
3 more terpenes selected from the group
4 consisting of geraniol, thymol, citral, carvone
5 (for example L-carvone), eugenol, b-ionone or a
6 mixture thereof.
7
- 8 15. A composition according to any preceding claim
9 wherein the terpene component comprises a
10 mixture of geraniol, thymol and eugenol.
11
- 12 16. A composition according to claim 14 wherein the
13 terpene component comprises 100% thymol.
14
- 15 17. A composition according to claim 14 wherein the
16 terpene component comprises 50% geraniol and
17 50% thymol w/w.
18
- 19 18. A composition according to claim 14 wherein the
20 terpene component comprises 50% eugenol and 50%
21 thymol w/w.
22
- 23 19. A composition according to claim 14 wherein the
24 terpene component comprises 33% geraniol, 33%
25 eugenol and 33% thymol w/w.
26
- 27 20. A composition according to claim 14 wherein the
28 terpene component comprises 33% eugenol, 33%
29 thymol and 33% citral w/w.
30

- 1 21. A composition according to claim 14 wherein the
2 terpene component comprises 25% geraniol, 25%
3 eugenol, 25% thymol and 25% citral w/w.
4
- 5 22. A composition according to claim 14 wherein the
6 terpene component comprises 20% geraniol, 20%
7 eugenol, 20% citral, 20% thymol and 20% L-
8 carvone w/w.
9
- 10 23. A composition according to any preceding claim
11 wherein the terpene component is associated
12 with a surfactant.
13
- 14 24. A composition according to any preceding claim
15 wherein the surfactant is selected from the
16 group consisting of sodium lauryl sulphate,
17 polysorbate 20, polysorbate 80, polysorbate 40,
18 polysorbate 60, polyglyceryl ester,
19 polyglyceryl monooleate, decaglyceryl
20 monocaprylate, propylene glycol dicaprylate,
21 triglycerol monostearate,
22 polyoxyethylenesorbitan monooleate, Tween®,
23 Span® 20, Span® 40, Span® 60, Span® 80, Brig 30
24 or a mixture of two or more thereof.
25
- 26 25. A composition according to any preceding claim
27 comprising 1 to 99% by volume terpenes, 0 to
28 99% by volume surfactant and 1 to 99% hollow
29 glucan particles or cell wall particles.
30
- 31 26. A composition according to claim 25 comprising
32 from about 10 to about 67% w/w terpenes, from

1 about 0.1 to about 10% w/w surfactant and from
2 about 40 to about 90% w/w hollow glucan
3 particles or cell wall particles.
4

5 27. A composition according to any preceding claim
6 suitable for killing bacteria or fungi.
7

8 28. A composition according to any preceding claim
9 suitable for killing mold.
10

11 29. A composition according to any preceding claim,
12 suitable for killing mycoplasma.
13

14 30. A composition according to any preceding claim
15 wherein the terpenes used are food grade.
16

17 31. A composition according to any preceding claim
18 comprising an additional food grade active
19 compound.
20

21 32. A composition according to claim 31 wherein the
22 additional food grade active compound is an
23 antimicrobial agent or enzyme.
24

25 33. A composition according to any preceding claim
26 comprising an antimicrobial agent, an anti-
27 fungal agent, an insecticidal agent, an anti-
28 inflammatory agent or an anaesthetic.
29

30 34. A composition according to any preceding claim
31 further comprising an antioxidant.
32

- 1 35. A composition according to claim 34 wherein the
2 antioxidant is rosemary oil, vitamin C or
3 vitamin E.
4
- 5 36. A composition according to any preceding claim
6 in the form of a dry powder.
7
- 8 37. A composition according to any one of claims 1
9 to 35 in a pellet, tablet or other solid form.
10
- 11 38. A composition according to any preceding claim
12 comprising a dispersal agent which promotes
13 dispersal of the composition when placed into a
14 liquid.
15
- 16 39. A composition according to any preceding claim
17 in combination with an agriculturally, food or
18 pharmaceutically acceptable carrier or
19 excipient in a liquid, solid or gel-like form.
20
- 21 40. A composition according to any one of claims 1
22 to 35 suspended or dissolved in a liquid.
23
- 24 41. A composition according to claim 40 wherein the
25 liquid is water.
26
- 27 42. A composition according to either claim 40 or
28 41 comprising from about 500 to about 10,000
29 ppm hollow glucan particles or cell wall
30 particles, where the particles contain from
31 about 1 to about 67% terpene component.
32

- 1 43. A composition according to claim 42 comprising
2 from about 1000 to about 2000 ppm hollow glucan
3 particles or cell wall particles, where the
4 particles contain from about 10 to about 50%
5 terpene component w/w.
6
- 7 44. A composition according to any one of claims 40
8 to 43 comprising between about 1 ppm and about
9 25 ppt of the terpene component.
10
- 11 45. A composition according to claim 44 comprising
12 between about 100 to 1000 ppm of the terpene
13 component.
14
- 15 46. A composition according to any one of claims 1
16 to 39 which is dispersed in water, saline,
17 aqueous dextrose, glycerol or ethanol to form a
18 solution or suspension.
19
- 20 47. A composition according to claim any preceding
21 claim which includes a wetting agent, an
22 emulsifying agent or a pH buffering agent.
23
- 24 48. A composition according to any preceding claim
25 dispersed in a liquid human or animal food or
26 drink material.
27
- 28 49. A composition according to any preceding claim
29 in a form suitable for oral administration.
30

- 1 50. A composition according to any one of claims 1
2 to 46 in a form suitable for parental
3 administration.
4
- 5 51. A composition according to any one of claims 1
6 to 46 in a form suitable for topical
7 administration.
8
- 9 52. A method of preparing a hollow glucan particle
10 or cell wall particle encapsulating a terpene
11 component, said method comprising the steps of;
12 a) providing a terpene component;
13 b) providing a hollow glucan particle or cell
14 wall particle;
15 c) incubating the terpene component with the
16 glucan particle or cell wall particle under
17 suitable conditions for terpene
18 encapsulation; and
19 d) recovering the hollow glucan particle or cell wall
20 particle encapsulating the terpene component.
21
22
- 23 53. A method according to claim 52 further
24 comprising the step of drying the glucan
25 particle or cell wall particle encapsulating
26 the terpene component.
27
- 28 54. A method according to claim 53 wherein drying
29 is achieved by freeze drying, fluidised bed
30 drying, drum drying or spray drying.
31

- 1 55. A method according to any one of claims 52 to
2 54 wherein in step a) the terpene component is
3 provided as a suspension in an aqueous solvent.
4
- 5 56. A method according to claim 55 wherein the
6 terpene component is provided in association
7 with a surfactant.
8
- 9 57. A method according to claim 56 wherein the
10 surfactant is polyoxyethylenesorbitan
11 monooleate at a concentration of about 0.1 to
12 10% by volume of the total reaction mixture.
13
- 14 58. A method according to any one of claims 52 to
15 54 wherein in step a) the terpene component is
16 provided as a true solution in the aqueous
17 solvent.
18
- 19 59. A method according to any one of claims 52 to
20 58 wherein in step b) the hollow glucan
21 particle or cell wall particle is provided as a
22 suspension in water or other suitable liquid.
23
- 24 60. A method according to claim 59 wherein the
25 suspension comprises approximately 1 to 1000 mg
26 glucan particle or cell wall particles per ml.
27
- 28 61. A method according to claim 59 wherein the
29 particles are dispersed in a volume of from the
30 hydrodynamic volume (HV) to 1.5HV of liquid.
31

1 62. A method according to any one of claims 52 to
2 58 wherein in step b) the hollow glucan
3 particle or cell wall particle is provided as a
4 dry powder.

5

6 63. A method according to any one of claims 52 to
7 62 wherein in step c) the reaction is carried
8 out at atmospheric pressure at a temperature of
9 about 20 to 37°C.

10

11 64. A method of killing a microorganism, said
12 method comprising the step of;
13 - contacting said microorganism with a
14 composition comprising a hollow glucan
15 particle or cell wall particle encapsulating
16 a terpene component.

17

18 65. A method of treating or preventing infection of
19 a plant, said method comprising the step of;
20 - administering, in a therapeutically
21 effective dose, a composition comprising a
22 hollow glucan particle or cell wall particle
23 encapsulating a terpene component to the
24 plant or to soil in proximity to the plant.

25

26 66. A method according to claim 65 wherein the
27 infection of the plant is caused by a nematode.

28

29 67. A method according to claim 65 wherein the
30 infection of a plant is caused by a fungus.

31

- 1 68. A method according to claim 67 wherein the
2 fungus is downy mildew, powdery mildew or
3 botrytis bunch rot.
4
- 5 69. A method according to any one of claims 65 to
6 68 wherein the plant is a grape vine.
7
- 8 70. A method according to any one of claims 65 to
9 69 wherein the composition is administered 21
10 days or less prior to harvest of a crop from
11 the plant.
12
- 13 71. A method according to claim 70 wherein the
14 composition is administered 14 days or less
15 prior to harvest.
16
- 17 72. A method according to claim 71 wherein the
18 composition is administered 7 days or less
19 prior to harvest.
20
- 21 73. A method according to claim 72 wherein the
22 composition is administered 3 days or less
23 prior to harvest.
24
- 25 74. A method according any one of claims 65 to 73
26 wherein the composition is administered by
27 spraying.
28
- 29 75. A method according to claim 74 wherein the
30 composition is sprayed at a rate of 500 L/Ha or
31 greater.
32

76. A method according to claim 75 wherein the composition is sprayed at a rate of 900 L/Ha or greater.
77. A method according to claim 76 wherein the composition is sprayed at a rate of 1200 L/Ha or greater.
78. A method according to any one of claims 65 to 73 wherein the composition is administered via irrigation.
79. Use of a composition comprising a hollow glucan particle or cell wall particle encapsulating a terpene component in the manufacture of a medicament for preventing or treating an infection in a patient, wherein said medicament is suitable for being administered to said patient in a therapeutically effective dose.
80. Use according to claim 79 wherein the infection of the patient is caused by *Staphylococcus aureus*, *Aspergillus Fumigatus*, *Mycoplasma Iowae*, *Penicillium* sp. Or *Mycoplasma Pneumoniae*.
81. Use according to claim 80 wherein the composition is administered orally, vaginally, rectally, by inhalation, topically or by parenteral routes.

82. A composition comprising a hollow glucan particle encapsulating a terpene component for use in the prevention or treatment of an infection in a patient or a plant.
83. Use of a hollow glucan particle encapsulating a terpene component in the manufacture of a medicament for the treatment of an infection in a plant.
84. The use of claim 83 wherein the infection is caused by *Aspergillius fumigatus*, *Sclerotinta homeocarpa*, *Rhizoctonia solani*, *Colletotrichum graminicola* or *Penicillium* sp.