Title: COMPOSITIONS COMPRISING A GERMINANT AND AN ANTIMICROBIAL AGENT

Abstract: The present invention provides an antiseptic composition comprising a germinant and an antimicrobial agent, wherein the germinant increases a pathogen’s susceptibility to attack from the antimicrobial agent and wherein the antimicrobial agent does not inhibit the germinant. The present invention also provides an antiseptic wipe, handwash or paint comprising such antiseptic composition and a method of sterilizing a surface using such a composition.
COMPOSITIONS COMPRISING A GERMINANT AND AN ANTIMICROBIAL AGENT

Field of the Invention
The present invention relates to compositions having antimicrobial properties and methods of disinfecting surfaces using such compositions. More specifically, the present invention relates to compositions which have effective antimicrobial activity against spore forming pathogens.

Background of the Invention
Spore forming pathogens, such as *C. difficile*, are important infectious agents. *C. difficile* is the leading cause of nosocomial diarrhoea in the UK and has been responsible for a significant number of deaths in UK hospitals. Recent figures show a decline in the number of cases in *C. difficile* infection. However, despite this decrease there are still nearly 10 times as many cases of *C. difficile* compared to MRSA. PCR ribotype 027 is now the most common ribotype isolated from *C. difficile* patients, followed by ribotype 106 and 001.

Infected patients excrete large numbers of *C. difficile* spores and cells into the environment which acts as a reservoir of infection. Patients therefore generally acquire the organism by ingestion of its spores from the contaminated environment. Once ingested the spores are able to survive the acidic environment of the stomach and pass into the GI tract where they germinate into vegetative cells to produce toxins A and B, which may cause disease in susceptible patients.

Spore germination is an irreversible process in which a highly resistant, dormant spore is transformed into a metabolically active cell. Germination occurs in several stages; the first being activated by the binding of germinant receptor on the plasma membrane. This is then followed by the loss of heat resistance and various cations including potassium, hydrogen and sodium and the complex of calcium and dipicolinic acid (DPA). This results in the partial re-hydration of the core, however the organism does not become fully hydrated until the cortex is degraded. As the cortex hydrolyses, the small acid soluble proteins are degraded and the metabolic activity of cell is resumed.
It is known in the art that *C. difficile* may be simulated to sporulate by exposure to germination solutions comprising a combination of glycine and taurocholate. The transition from the spore state causes the pathogens to be susceptible to attack from antibacterial agents, such as chlorhexidine. The aim of the prior art has been to design germinate/exterminate protocols which take advantage of the susceptibility of the pathogen following germination. Such a protocol is shown in Figure 1. Specifically, attempts have been made in the prior art to design antibacterial solutions which stimulate germination and which have an antibacterial agent to attack the germinated pathogens. However, the antibacterial agents used in such approaches have an inhibitory effect on the germinating solution such that germinate/exterminate approach has had limited success in the clinical setting.

The aim of the present invention is there to provide an alternative antimicrobial composition which overcomes these problems and which is effective against clinically important pathogens.

**Summary of the Invention**

The inventors of the present invention have found that particular compositions comprising combinations of amino acids with sodium taurocholate have enhanced sporulating activity compared to the prior art compositions of glycine only in combination with taurocholate. Furthermore, the inventors have discovered that particular antimicrobial agents are able to kill the germinated spores effectively whilst acting as a preservative in the composition. These agents do not have an inhibitory effect on the germination of the pathogen observed in the prior art antimicrobials used in the germinate/exterminate approach.

Furthermore, the inventors of the present invention have found that, as well as stimulating the formation of vegetative cells from spores of *C. difficile*, the compositions of the present invention prevent sporulation of vegetative cells of *C. difficile*. This is particularly advantageous in the clinical environment. Many vegetative cells (as well as spores) are excreted in the faeces of hospitalised patients. The vegetative cells will eventually sporulate and the spores then act as a vector of transmission of disease. Keeping the cells in the vegetative form is preferred since they will be naturally killed by air, as they are strictly anaerobic, or
common hard surface disinfectants used in hospitals. The compositions of the present invention therefore have a two way attack; first, converting pores into vegetative cells and, second, holding vegetative cells in the vegetative form.

In a first aspect, the present invention provides an antiseptic composition comprising a germinant and an antimicrobial agent, wherein said germinant increases a pathogen's susceptibility to attack from the antimicrobial agent and wherein the antimicrobial agent does not inhibit the germinant.

In a second aspect, the present invention provides an antiseptic wipe comprising a fabric, mesh or gauze type material impregnated with a composition of the invention. In a third aspect, the present invention provides an antiseptic hand wash or paint for coating on an abiotic surface which handwash or paint comprises a composition of the invention.

In a fourth aspect, the present invention provides a method of disinfecting a surface such that it is essentially free of pathogens, the method comprising contacting the surface with the composition of the invention or an antimicrobial wipe or paint comprising a composition of the invention for a time sufficient for the pathogens to germinate and be killed.

The antiseptic composition of the present invention comprises a germinant in combination with an antimicrobial agent. The germinant is selected from at least two amino acids. Preferably the amino acids are the combination of histidine, arginine, aspartic acid, glycine. More preferably the combination of amino acids is histidine, arginine, aspartic acid, glycine and valine. This particular combination of amino acids has been shown by the inventors to be particularly advantageous having increased germination effects on bacterial spores.

The germinant is provided in an amount suitable to stimulate effective sporulation of the spores. Preferably, the germinant is provided in an amount of from 1 to 10mM based on the final composition. The constituent amino acids within the germinant solution may be provided in an amount of 50mM (histidine), 50mM (glycine), 50mM (arginine), 50mM 1:50 (valine) and 50mM (aspartic acid).
The antimicrobial agent is an agent which is effective at killing a pathogenic microorganism upon its contact with a surface on which the pathogen is found. Preferably, the antimicrobial agent is effective or killing the pathogen when it is in the germination state following sporulation. The antimicrobial agent may also be effective at killing pathogens which do no form spores.

Preferably, the antimicrobial agent is a combination of benzalkonium chloride and benzyl alcohol. The ratio of benzalkonium chloride to benzyl alcohol in the antiseptic composition is 1:50. The antimicrobial agent is provided (with respect to the total amount of antimicrobial agent in the antiseptic composition) in an amount of from 0.01% to 2%.

The antiseptic composition may be provided in a form required for use to disinfect surfaces in the clinical environment. Preferably such surfaces are abiotic surfaces. Alternatively, the surface may be the skin of a patient, doctor or carer. Preferably, the composition is provided in the form of a liquid, gel, foam or spray which may be conveniently applied to a surface requiring treatment. In a further embodiment, an antimicrobial wipe is provided comprising a fabric, mesh or gauze type material impregnated with the antiseptic composition. The composition may also be provided in tablet form. Such tablet forms may be dissolved in a suitable amount of water and the resultant solution used as a disinfecting agent by hospital cleaners, for example, in cleaning large surface areas. In the embodiment where the surface is a person's skin, then the antiseptic composition may be in the form of a handwash or wipe. Preferably, the antiseptic composition is in the form of a paint which may be painted on to the surface to be treated. Such a paint is particularly advantageous since once dried it is effective at killing pathogens over a longer period of time compared to prior art antiseptics which are ineffective once dried and which the antimicrobial agent leads to inhibition of the germinating agent after a particular length of time. Even more preferably the antiseptic is in the form of a lacquer which forms a hard film upon the surface to which it is applied and is not readily wiped off.

The antiseptic compositions of the present invention are generally capable of sanitising surfaces from a broad spectrum of pathogens. The composition is
particularly effective against spore forming bacteria. An example of such a spore forming bacteria is *C. difficile*. However, the composition may also be effective against other pathogens such as MRSA, *P. aeruginosa*, *C. albicans* and *A. niger*. Following is a description, by way of example only, with reference to the accompanying figures and table, of a method of putting the invention into effect, wherein:

Figure 1 schematically shows a germinate/exterminate protocol used in the prior art.

Figure 2 shows the log reduction in CFU/mL of *C. difficile* NCTC 11204 spores exposed to different germinant solutions for one hour, followed by heat shocking for 10 minutes.

Figure 3 shows log reduction in CFU/mL of *C. difficile* NCTC 11204 spores after one hour's exposure to each amino acid germinant solution, followed by heat shocking for 10 minutes at 70°C.

Figure 4 shows the effect of an amino acid combination (arginine, aspartic acid, glycine and histidine) concentration in a germinant solution also containing 6.9mM sodium taurocholate after 1 hour incubation with *C. difficile* spores of ribotype 027, 001, 106 and strain NCTC 11204.

Figure 5 shows the log reduction in CFU/mL of spores of different strains of *C. difficile* after one hour exposure to different germinant solutions, followed by 10 minutes heat shock.

Figure 6 shows the effect of pH on the germination of *C. difficile* NCTC 11204 spores in Tris buffer containing 10mM arginine, aspartic acid, histidine and glycine and 6.9mM sodium taurocholate.

Figure 7 shows the log reductions in CFU/mL of *C. difficile* spores after 1hr exposure to different germinant solutions, followed by either heat shock or chilling
on ice (AA= amino acids, ST= sodium taurochoiate, BZC= benzaikonium chloride, BZA= benzyl alcohol).

Figure 8 shows the log reductions in CFU/mL of C. difficile spores after 1hr exposure to different germinant solutions, followed by either heat shock or chilling on ice (CHG= chlorhexidine digluconate).

Figure 9 shows the count of C. difficile vegetative cells in PBS (control) culture. 100% of the count is spores after 24 hrs

Figure 10 shows the count of C. difficile vegetative cells in a germination solution of the invention. After 72 hrs 100% of the count is still vegetative cells.

Table 1 shows the log reductions in CFU/mL of C. difficile spores after 1hr exposure to different germinant solutions, followed by either heat shock or chilling on ice.

**Example 1 - combinations of amino acids for germinant solution**

**Strains of C. difficile**

A ribotype 106 strain (18587), a ribotype 001 strain (8565) (Health Protection Agency, North East UK) and the NCTC 11204 reference strain were used together with three ribotype 027 strains; R20291 (Anaerobic Reference Laboratory, Cardiff), 18040 and 15900 (HPA, North East UK).

**Preparation of C. difficile spore suspensions**

Spores of C. difficile were prepared as previously described (Shetty et ai, 1999). A blood agar plate was inoculated with the relevant strain of C. difficile and incubated anaerobically (MiniMACS anaerobic work station, Don Whitley, Shipley, UK), for 72 hours, at 37°C. The plate was then removed and left in air, at room temperature for 24 hours, before harvesting all colonies and placing in 10mL of 50% saline (0.9% w/v) and 50% methylated spirits. The suspension was vortex mixed thoroughly before being filtered through glass wool and was stored at 4°C until use. Before each experiment spore suspension were centrifuged at 13000rpm for 5 minutes and resuspended in sterilised distilled water (SDW).
**Amino acids**
The following amino acids were used in the experiments detailed below: glycine, L-
isoleucine, L-proline, D-alanine, L-glutamic acid, L-erine, L-threonine, L-
spartic acid, L-arginine, L-valine, L-leucine, L-glutamine, L-
tryptophan and L-asparagine (all from Sigma-Aldrich, UK), L-
lysine, L-histidine and L-methionine (BDH), L^-phenylalanine (Fisons) and L-
cysteine (Fluka). Tyrosine was not tested as it did not dissolve in distilled water.

**Efficacy of different amino acids as co-germinants against the spore of C. difficile**
All germinant solutions contained 0.4% (w/v) of the amino acid (concentration of
glycine used in previous study (Wheeldon et al., 2008)) and with 13.8mM (w/v)
sodium taurocholate (Sigma-Aldrich, UK), (double the physiological concentration
in the duodenum (Leverrier et al., 2003)), which were dissolved in distilled water
and sterilised by autoclaving at 121°C for 15 minutes. Double strength
thioglycollate medium (Oxid, UK), containing 13.8mM sodium taurocholate (ST)
was also tested for comparison against the other germinant solutions. One
hundred microlitres of each germinant solution was then added to 100μL if
C.difficile spores (containing 10^6 CFU/mL) and was vortex mixed thoroughly. After
one hour the entire 200μL volume was added to 9.8mL of Wilkins Chalgren broth
(Oxoid, UK), equilibrated to either 70°C for 10 minutes or chilled on ice. After
appropriate dilution, 1ml_ samples were placed in sterile Petri dishes before the
addition of 15mL of molton Fastidious Anaerobe Agar (FAA) (Lab M, UK),
containing 0.1 % (w/v) ST and 5% (v/v) defibrinated horse blood. The plates were
mixed thoroughly before being incubated anaerobically for 48 hours, at 37°C. This
method was adapted from Levinson and Hyatt (1966).

**Determination of the optimum concentration of amino acids required for the
germination of C. difficile spores**
Arginine, aspartic acid, glycine and histidine were prepared at 200, 20, 2, 0.2 and
0.02mM, together with 13.8mM ST (which is double the strength required as they
are diluted 1:2 during the experiment) and were dissolved in distilled water. All
solutions were heated until dissolved and were sterilised by autoclaving. The same method as above was used to test the different concentrations of germinant solutions against the spores of *C. difficile* NCTC 11204, ribotype 027 (R20291), 001 and 106.

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**Efficacy of amino acids and taurocholate against the spore of six different strain of *C. difficile***

Arginine, aspartic acid, glycine and histidine (at 20mM w/v) were added separately or in combination to 13.8mM (w/v) ST and dissolved by heating in distilled water, before sterilisation by autoclaving. The same method as described above was used to determine the efficacy of these amino acid solutions against the spores of *C. difficile* ribotype 001, 106, 027 (R20291, 15900 and 18040) and NCTC 11204.

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**Effect of pH of buffered germinant solutions on the germination of *C.difficile***

spore

Arginine, aspartic acid, glycine and histidine (at 20mM w/v), 13.8mM (w/v) ST and 200mM (w/v) Tris was dissolved in distilled water and adjusted to pH 5, 6, 7, 8 and 9 using hydrochloric acid before sterilisation by filtration (0.2μm) or autoclaving. The same method as above was used to test each buffered solution against the spores of *C. difficile* NCTC 11204.

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**Example 2 - Effect of germinant solution with antimicrobial and preservative on *C.difficile***

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**Germinant solution**

A solution containing 100mM (w/v) glycine, histidine, valine, aspartic acid and arginine, 13.8mM (w/v) sodium taurocholate, 100mM (w/v) Tris buffer, 0.04% (w/v) benzalkonium chloride, 2% (v/v) benzyl alcohol was made up to the required volume with distilled water and adjusted to pH 7 using hydrochloric acid. The solution was heated and mixed thoroughly to dissolve all of the components before filtration through a pore size of 0.2μm for sterilization. (This solution is double the working strength as it is diluted 1:2 during the experiment).
Preparation of *C. difficile* spore suspensions

Preparations of *C. difficile* spores were prepared as noted above.

Neutraliser

The neutraliser was adapted from Espigares et al., (2003) and contained the following: 120mL (v/v) Tween 80, 25mL (v/v) of 40% sodium metabisulphite, 15.69g (w/v) sodium thiosulphate pentahydrate, 10g (w/v) L-cysteine, 4g (w/v) lecithin and made up to 1000mL with distilled water. The pH was adjusted to 7 and was autoclaved to sterilize.

Germination efficacy testing

One hundred microlitres of the germinant solution (as described above) was added to 100μL of *C. difficile* spores (containing 10⁶ CFU/mL) and was vortex mixed thoroughly. For the control, 100μL of sterilized distilled water was mixed with 100μL of *C. difficile* spore suspension. Spore suspensions were also incubated with solutions containing benzaikonium chloride and Tris buffer (without amino acids or taurocholate), benzyl alcohol with Tris buffer (without amino acids and taurocholate) and a solution containing the five amino acids and taurocholate (with no antimicrobials). After one hour the entire 200μL volume was added to 9.8mL of neutraliser (as described above), equilibrated to either 70°C for 10 minutes (heat shock to kill germinating cells) or chilled on ice (controls). After appropriate dilutions, 1mL samples were placed in sterile petri dishes before the addition of 15mL of molten Fastidious Anaerobe Agar (FAA) (Lab M, UK), containing 0.1% (w/v) ST and 5% (v/v) defibrinated horse blood. The plates were mixed thoroughly before being incubated anaerobically for 48 hours, at 37°C (miniMACS Anaerobic Workstation (Don Whitley Scientific Ltd)). This method was adapted from a previous study by Levinson and Hyatt (1966).

Results and discussion

Efficacy of other amino acids as co-germinant with glycine against the spores of *C. difficile* NCTC 11204

Incubation of *C. difficile* NCTC 11204 spores with arginine, aspartic acid or histidine with glycine and ST produced large log reductions in CFU/mL of 2.78 (99.8%), 3.06 (9.99%) and 3.12 (99.9%) respectively after heat shocking. Valine (with glycine
and ST) and leucine (with glycine and ST) produced log reductions in CFU/mL of 1.72 (98.1%) and 1.62 (97.6%) respectively, which were similar to that of glycine and ST alone (1.85 log reduction (98.6%)). All other amino acids tested produced log reductions smaller than that of glycine and sodium taurocholate alone.

Efficacy of co-germinants alone and in combination with glycine and sodium taurocholate against the spore of *C. difficile* NCTC 11204

When the four most effective amino acids from the initial screening (arginine, aspartic acid, histidine and glycine) were placed together in solution with ST and incubated with *C. difficile* spores, a greater log reduction in CFU/mL of 3.71 (99.9%) was produced after heat shocking, compared with when spores were incubated with either arginine, aspartic acid or histidine with ST, but without glycine there was less than one log reduction produced by each solution.

Determination of the optimum concentration of amino acids required for the germinations of *C. difficile* spores

*Clostridium difficile* spore germination was optimum in all strains tested when the amino acid concentration was between 10 and 100mM. Below this concentration, germination of *C. difficile* spores was significantly reduced with less than a one log reduction observed at 0.1 mM and below. In all strains of *C. difficile* there was a significant reduction in CFU/mL observed when spores were exposed to 100mM of amino acids without heat shocking.

Efficacy of amino acids and sodium taurocholate against the spores of different strains of *C. difficile* PCR ribotype 027

All strains of *C. difficile* PCR ribotype 027 exposed to the four amino acids in combination with ST produced log reductions similar to those produced when spores were exposed to thioglycollate medium and ST. Both aspartic acid and histidine increased the log reduction in CFU/mL observed in each ribotype 027 strain when combined with glycine and sodium taurocholate, however, arginine did not cause any further increase in log reduction in CFU/mL when added to glycine and ST. Most interestingly, the two clinical strains (18040 and 15900) gave significantly larger log reductions in response to the thioglycollate medium or the four amino acids combined with ST compared with the R20291 strain.
Efficacy of amino acids and sodium taurocholate against the spores of six different strain of *C. difficile*

The greatest log reductions in CFU/mL were observed with the NCTC 11204 reference strain of *C. difficile* after exposure to the amino acid and thioglycollate solutions. PGR ribotype 001 gave very similar log reduction values to the R20291 strain of PCR ribotype 207 in each of the different combination of germinant solution tested. For example, log reductions in CFU.mL of 2.12 (99.2%) and 1.98 (99%) were produced in *C. difficile* ribotype 001 and 027 R20291 respectively, after incubation with the four amino acids and ST, followed by heat shock.

Effect of pH of buffered germinant solutions on the germination of *C. difficile* NCTC 11204 spores

The pH of the buffered germinant solution greatly affected the log reduction in CFU/mL produced after incubation with the amino solution (and ST), followed by heat shock. At an acidic pH of 5, the germination solution did not produce any log reductions in CFU/mL, where as a 3.32 log reduction in CFU/mL (99.9%) was observed at a neutral pH of 6.98; which was found to be the optimum pH. A more alkaline pH of 8.81 did decrease the log reduction in CFU/mL observed, but to a much smaller extent than that of an acidic pH.

Effect of germinant solution with antimicrobial and preservative on *C. difficile*.

Over a 3 log reduction in CFU/mL (99.9% reduction) was produced after *C. difficile* spores were incubated with a mixture of amino acids and taurocholate in Tris buffer for 1 hour followed by heat shocking. However, without heat shocking there was very little reduction (0.13 log reduction) in the number of remaining spores after incubation with the amino acid solution. Incubation of *C. difficile* spores with a solution containing benzalkonium chloride in Tris or benzyl alcohol in Tris induced a very low log reduction in CFU/mL of less than 0.3 both with and without heat shocking. However, when *C. difficile* spores were incubated with a solution containing amino acids, taurocholate, benzalkonium chloride and benzyl alcohol in Tris, there was a 2.77 log reduction in CFU/mL (99.8% reduction) with ice treated
spores and a 2.99 log reduction in CFU/mL (99.9% reduction) with heat shocked spores.

The inventors have also shown that EDTA and chlorohexidine inhibit the germinant solution, where as benzalkonium chloride and benzyl alcohol do not affect germination. Benzalkonium chloride and benzyl alcohol were not sporicidal and did not initiate germination. Non heat-treated spores were killed after 1hr incubation with benzalkonium chloride, benzyl alcohol, amino acid and sodium taurocholate and spores appear to be killed as they germinate with the germinant solution containing benzalkonium chloride and benzyl alcohol.

The neutraliser was found to be non-toxic towards C. difficile cells and other bacteria and was effective at nullifying the activity of the antimicrobials. Therefore the reduction in CFU/mL produced after 1hr incubation with benzalkonium chloride, benzyl alcohol, amino acid and sodium taurocholate is not likely to be the result of a 'carry over' effect on the agar plates.

The present inventors have also found that benzalkonium chloride and benzyl alcohol do not affect the efficacy of the germinant solution. Benzalkonium chloride and benzyl alcohol therefore show preservative efficacy and appear to kill germinating spores of C. difficile.

Inhibitory effect of germination solution on spore formation

The results shown in Figures 9 and 10, indicate that after 72 hrs of culture in the presence of the germination solution 100% of the count is still vegetative cells whilst in the control (cultured cells in PBS), 100% of the count are spores after 24 hrs. These results demonstrate that the germination solution of the invention prevents sporulation in vegetative cells of C. difficile.
Claims

1. An antiseptic composition comprising a germinant and an antimicrobial agent, wherein the germinant increases a pathogen's susceptibility to attack from the antimicrobial agent and wherein the antimicrobial agent does not inhibit the germinant.

2. The composition of claim 1, wherein the germinant comprises a salt of taurocholate and at least two amino acids.

3. The composition of claim 2, wherein the salt of taurocholate is sodium taurocholate and the at least two amino acids are histidine, arginine, aspartic acid, glycine and valine.

4. The composition of any preceding claim, wherein the bacterial pathogen is a pathogen selected from *C. difficile*, methicillin resistant *Staphylococcus aureus* (MRSA), *E.coli*, *Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus niger*.

5. The composition of any preceding claim wherein the antimicrobial agent comprises benzylkonium chloride and benzyl alcohol.

6. The composition of any preceding claim, wherein the germinant is provided in an amount of from 1 to 10mM based on the final composition and the antimicrobial agent is provided in an amount of from 0.01 % to 2%.

7. The composition of any preceding claim in the form of a liquid, gel, foam, spray or tablet.

8. An antiseptic wipe comprising a fabric, mesh or gauze type material impregnated with a composition according to any preceding claim.

9. An antiseptic hand wash, paint or lacquer for coating an abiotic surface, comprising a composition according to any of claims 1-7.
10. A method of sterilising a surface such that it is essentially free of pathogens, the method comprising contacting the surface with the composition according to any of claims 1-7 or an antiseptic wipe according to claim 8 or a paint according to claim 8 for a time sufficient for the pathogens to germinate and be killed.
Figure 1

- Spores
- Germination solution
- Vegetative cells
- RESISTANT
- HEAT
- Susceptible
Figure 2
Figure 3
Figure 7

Germinant solution

Log reduction in CFU/mL

- BZC + Tris
- BZA + Tris
- AA + ST
- AA + ST + BZC + Tris
- AA + ST + BZC
- AA + ST + BZC + Tris
- BZA + Tris

Ice
Heat
Figure 8

Germinant solution

Log reduction in CFU/mL

- EDTA, AA + ST
- BZC, AA + ST
- BZC, CHG, AA + ST
- BZC, BZA, AA + ST

Ice
Heat
**Figure 10**

- **Log CFU/ml**
- **Time in Germinant (Hours)**

- Total viable count (spores and vegetative cells)
- (Of which are vegetative cells)
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<thead>
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<th>Germination solution</th>
<th>Log reduction in CFU/mL after 1hr incubation followed by chilling on ice</th>
<th>Log reduction in CFU/mL after 1hr incubation followed by heat shocking</th>
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<tbody>
<tr>
<td>Benzalkonium chloride and Tris</td>
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<td>Benzyl alcohol and Tris</td>
<td>0.16</td>
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<td>Amino acids, taurocholate, benzalkonium chloride, benzyl alcohol and Tris</td>
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Table 1
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

International application No
PCT/GB2011/050278
INV. A01N41/04 A01P1/00 A01N33/12 A01N45/00 A47K10/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC.

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A01N A47K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tbody>
<tr>
<td>X</td>
<td>WO 03/06 1610 AI (WALKER EDWARD B [US]) 31 July 2003 (2003-07-31) page 2, line 8 - line 12 page 10, line 25 - line 26 page 11, line 10 - page 12, line 5 page 12, line 3 - line 5 page 12, line 21 - page 13, line 2 page 14, line 24 - page 15, line 2 page 18, line 4 - page 19, line 16 example El ----</td>
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X Further documents are listed in the continuation of Box C. X See patent family annex.

* Special categories of cited documents:
  *"A" document defining the general state of the art which is not considered to be of particular relevance
  *"E" earlier document but published on or after the international filing date
  *"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  *"O" document referring to an oral disclosure, use, exhibition or other means
  *"P" document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search 17 May 2011
Date of mailing of the international search report 06/07/2011

Name and mailing address of the ISA/Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk
Tel. (+31-70) 345-2040, Fax. (+31-70) 340-3016
Habermann, Jörg
### Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No.
--- | --- | ---
X | US 6 656 919 BI (BAUGH CLARENCE L [US] ET AL) 2 December 2003 (2003-12-02) col umn 1, line 18 - line 25 | 1,4,6,7, 10
Y | col umn 4, line 49 - line 65 | 2,3
 | col umn 5, line 14 - line 18 | col umn 5, line 48 - line 62 | col umn 8, line 4 - line 22 | col umn 8, line 25 - line 62 | examples 3, 6
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