



Large-scale production method for Cordyceps and Ganoderma

Field of the invention

- 5 The present invention relates to the large-scale production of fungi, in particular to Cordyceps and/or Ganoderma, mycelium, spores and paste.

The present invention further relates to an apparatus and growth parameters for the large-scale production of the said fungi, and also to culture media, as well
10 as methods for the production of the fungi. The present invention also relates to extraction methods for the active ingredients of Cordyceps and Ganoderma. The invention also relates to the preparation of beverage and food comprising the fungi and/or fungi paste prepared according to the invention. In particular, it is provided a dairy product comprising milk and said fungi and/or fungi paste.

15

Background of the invention

Cordyceps, a family of fungi e.g. *Cordyceps Sinensis* (known as Dong Cong Xia Cao in Mandarin), is considered as one of the superior Chinese herbs in
20 Traditional Chinese Medicine (TCM), believed to be useful for enhancing immunity, reducing phlegm, strengthening lungs, fortifying kidneys and improving sexual vitality. Cordyceps strains grow on the body of a host caterpillar or insect larvae, which becomes mummified by an internal mat of Cordyceps mycelium. Eventually, a large, clavate stroma, sometimes as much
25 as 10-15 cm in length, grows out from the mummified host and bears an immersed perithecia in its upper part.

Ganoderma lucidum (Red LingZhi in Mandarin), is another family of fungi also considered a superior herb in TCM. It is believed to be capable of preventing
30 cancer, lowering cholesterol and to be therapeutic against heart and liver diseases. Fruiting body and spores of the LingZhi mushroom are the major forms prescribed in TCM. Other strains of LingZhi used may include



Ganoderma japonicum (Purple LingZhi) and *Ganoderma tsugae*, both of which are supposed to have therapeutic effects different from those of Red LingZhi.

5 Wild Cordyceps and Ganoderma can possess high concentrations of heavy metals and can also carry microbial contamination. It is therefore difficult to ensure the quality of such harvested wild growths. Industrially produced Cordyceps and Ganoderma, the qualities of which are controllable by the isolation and selection of pure strains and by the choice and amount of substrates added to the growth medium, have so far been mainly confined to
10 agar cultures or shake flask cultures which are not suitable for large-scale production. While a shake flask culture may easily grow the fungi, a scaled up production such as those using bio-reactors requires stringent control of parameters such as oxygen content, agitation method and intensity, temperature, amount of growth substrates such as carbon and nitrogen sources
15 and so on, and the optimal ranges of such parameters are not easily determined. Results have shown that static/shake flask liquid culture yields only 1% growth (where yield of the biomass is defined as dry mycelia per 100 ml of fermentation broth/liquid for a period of 45 to 72 hours). A reasonable period of time for the culture of a static/flask cultivation is at least two times longer than
20 that of a submerged fermentation system. Some strains such as *C. Gracilis*, *C. ophioglossoides*, *C. militaris* cannot even grow properly in a static/shake liquid culture. Furthermore, *Ganoderma* cells tend to be pelletised in a submerged fermentation system, especially one using a potato-dextrose medium such as the one recommended in US5,334,704, which results in affected hyphal growth.

25

Besides the fruiting bodies of Ganoderma, the spores of Ganoderma and Cordyceps would be extremely valuable in TCM. Unfortunately, Ganoderma and Cordyceps, while they can be grown to some extent in standard growth medium under standard conditions, have never been successfully cultured to
30 produce basidiospores. Consequently, large-scale production of the spores has not been achieved so far.

Summary of the invention

In a first aspect, the present invention provides in general terms an apparatus for large-scale production of Cordyceps and/or Ganoderma, preferably
5 comprising a bio-reactor. In particular, if used to culture Cordyceps, it can produce a yield of at least 5%, and if used to culture Ganoderma, it produces a yield of at least 3%.

In another aspect, the invention provides a method for the multi-stage liquid
10 fermentation, leading to a yield of at least 5% of Cordyceps or a yield of at least 3% of Ganoderma.

In another aspect, the invention provides a method of producing spores of Ganoderma and/or Cordyceps. In particular, the method comprises the step of
15 applying a pressure of 2 bar and higher to a culture of Ganoderma and/or Cordyceps during the growth phase.

In another aspect, the invention provides a novel medium for the culture of Cordyceps and/or Ganoderma.
20

In another aspect, the invention provides a medium for the culture of Cordyceps and/or Ganoderma fortified with sodium nitrate and/or Riboflavin (vitamin B2). In particular, the invention provides a medium for the culture of Cordyceps *militaris* or Ganoderma *japonicum* fortified with sodium nitrate and/or Riboflavin (vitamin
25 B2).

In another aspect, the invention provides a method comprising hot water extraction of the active ingredients of Cordyceps and/or Ganoderma.

30 In another aspect, the invention provides using Ganoderma spores (for example, *lucidum* spores), Cordyceps spores, Ganoderma mycelia and/or Cordyceps mycelia and Ganoderma paste and/or Cordyceps paste produced by

the process or system provided according to any embodiment of the invention as nutritional supplements.

5 In another aspect, the invention provides unicellular Ganoderma and/or Cordyceps. The invention further provides cultures of Ganoderma and/or Cordyceps in substantially unicellular form. The invention further provides cultures comprising 100% of unicellular culture of Ganoderma and/or Cordyceps.

10 In another aspect, the invention provides beverage and food comprising any Ganoderma and/or Cordyceps mycelium, spore or paste prepared in the presence of the medium according to the present invention. In particular, the invention provides milk fortified with at least one of Ganoderma mycelium, Cordyceps mycelium, Ganoderma spores, Cordyceps spores, Ganoderma
15 paste and Cordyceps paste produced in presence of the medium of the invention.

In another aspect, the invention provides a pharmaceutical composition comprising at least one of Ganoderma mycelium, Cordyceps mycelium,
20 Ganoderma spores, Cordyceps spores, Ganoderma paste and/or Cordyceps paste, produced in presence of the medium of the invention.

The invention also provides the use of Ganoderma mycelium, Cordyceps mycelium, Ganoderma spores, Cordyceps spores, Ganoderma paste and/or
25 Cordyceps paste, produced in presence of the medium of the invention, for the manufacture of a pharmaceutical composition, beverage (for example milk) and/or food (for example, nutritional supplement) for use in therapy. Therapeutic uses include, but are not limited to, treatment of tumors, stimulation of macrophages activity, or stimulation of immunological system, stimulation of
30 kidney and respiratory functions, enhance the circulatory function of the body, and others. Accordingly, the invention also provides a method for therapeutic treatment, in particular (but not limited to) for the treatment of tumors,

stimulation of macrophages activity, or stimulation of immunological system, stimulation of kidney and respiratory functions, enhance the circulatory function of the body, and others comprising the administration to a patient of: at least Ganoderma mycelium, Cordyceps mycelium, Ganoderma spores, Cordyceps spores, Ganoderma paste and/or Cordyceps paste, produced in presence of the medium of the invention, or a pharmaceutical composition, beverage (for example milk) and/or food (for example, nutritional supplement) comprising at least Ganoderma mycelium, Cordyceps mycelium, Ganoderma spores, Cordyceps spores, Ganoderma paste and/or Cordyceps paste, produced in presence of the medium of the invention.

Brief description of the figures

Preferred features of the invention will now be described, for the sake of illustration only, with reference to the following figures in which:

Figure 1 is a chart showing yield of dry weight mycelium versus concentration of different ingredients. The experiment was carried out by using shake flask fermentation.

Figure 2 is a chart showing effect of maltose on the yield of *Cordyceps*. The experiment was made by submerged fermentation in 75 l bio-reactor. The added minerals were: KH_2PO_4 of 1.5 g/L, and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ of 0.3 g/L.

Figure 3 is a chart showing the effect of aeration rate on the yield of *Cordyceps*. The experiment was made by submerged fermentation in 75 l bio-reactor.

Figure 4 is a bar graph showing the effect of stirring speed on the yield of *Cordyceps*. The experiment was made by submerged fermentation in 75 l bio-reactor

Figure 5 shows a microscopic observation of spores of *Ganoderma* produced by liquid cultivation.

Figure 6 shows profiling of proteins, glycoproteins and polysaccharides found in the active ingredients of *Ganoderma lucidum* and *Cordyceps sinensis* by SDS-PAGE.

Figure 7 shows profiling of proteins, glycoproteins and polysaccharides found in the active ingredients of *Ganoderma lucidum* and *Cordyceps sinensis* by Western Blotting.

Figure 8 shows a microscopic observation of mycelia and spores of *Cordyceps sinensis* obtained according to the invention.

Figure 9 shows a microscopic observation of mycelia and spores of *Cordyceps sinensis* obtained according to the invention.

Figure 10 relates to the growth of *Cordyceps militaris* in slant/plate agar in presence of YM medium (see Table 2) according to the increase of the temperature. The slant/plate on the left side of the photo relates to the growth at a temperature of 20°C, the slant/plate in the middle relates to a temperature of 25°C, and the slant/plate of the right side relates to a temperature of 29°C.

Figure 11 is an outline of the general method for the preparation of fortified milk according to the invention.

25

Detailed Description of the Invention

In the prior art, liquid medium growth of *Cordyceps* or *Ganoderma* has been restricted to static or shake culture employing, for example, an Erlenmeyer flask or the like on a shaker. A generic multi-stage liquid fermentation method using a shake flask culture has been disclosed in US 5,334,704. However, fundamental differences in theory and practice between the large-scale bio-reactor

30

production of the present invention and the prior art static/shake liquid bench top culture means that the prior art cannot provide successful mass production of the fungi using a direct scale-up of the shake culture. No matter how many stages are used, mass production of the said fungi is unachievable using bench top fermentation methods. Some strains of *Cordyceps* such as *C. Gracilis*, *C. ophioglossoides*, *C. militaris* cannot grow properly in a static/shake liquid culture due to un-optimisable parameters such as aeration, stirring and mixing effects because of the simplicity of glassware design and stirring methods of bench top apparatus.

10

In a shake flask culture the growth of the cells increases the viscosity of the liquid medium and thus impedes optimal aeration. This is a factor preventing high yield growth of *Cordyceps*.

15 The invention provides an apparatus, a growth medium and a method for submerged aerobic fermentation/cultivation growth of *Cordyceps* and *Ganoderma* species. The growth medium and method provided by this embodiment can eliminate, or at least reduce substantially, the level of heavy metal contamination and unwanted microbes (compare Table 8 and Table 9
20 with Table 7). Selected pure strains may be used for production and biotechnological parameters are carefully controlled such that authenticity and consistency of quality of the products can be ensured. Further, the invention provides an apparatus comprising a bio-reactor vessel shaped for large scale production comprising stirring means in order to stir continuously and regularly
25 the culture medium. This improves the rate and efficiency of aeration and consequently the delivery of oxygen to active cells, and can prevent the cells from lumping or settling down to the bottom of the vessel. As a result, *Ganoderma* and/or *Cordyceps* produced according to the present invention are substantially in unicellular form. On the contrary, the *Ganoderma* and
30 *Cordyceps* of the prior art, are in pluri-cellular form.

Pluri-cellular cultivation is related to sexual reproduction and/or cell differentiation. Like human, the starting point is a sperm-fertilized egg, and growing up through cell differentiation to develop many different cell lines so as to form a body eventually containing multi-organs, namely "a pluri-cellular culture".

Accordingly, the invention provides unicellular Ganoderma and/or Cordyceps. A qualitative analysis to show the unicellular nature of Ganoderma and/or Cordyceps can be seen in Figures 9 and 10, which shows the morphological data of cytology. The invention further provides cultures of Ganoderma and/or Cordyceps in substantially unicellular form. The invention further provides cultures comprising 100% of unicellular culture of Ganoderma and/or Cordyceps.

Accordingly, the present invention provides an apparatus for large scale production of fungi, comprising:

- a bio-reactor; and
- a liquid medium including water having at least one carbon source and a volume of at least 500 litres and a pH of at least pH 5; and
- means for agitating the liquid medium; and
- means for maintaining the temperature of the liquid medium at a temperature of at least 17°; and
- means for aerating the liquid medium at a rate of at least 1:0.5 v/v/m; and
- anti foaming agent.

The means for agitating is for example a stirrer. The stirring speed is at least 100 rpm. The liquid medium including water having at least one carbon source may be any medium described according to any embodiment of the present invention. The liquid medium may further comprise yeast extract, in particular yeast extract having a concentration of at least 15 g/l. The liquid medium may further comprise a peptone source, for instance having a concentration of at least 15 g/l. The liquid medium may comprise glucose as one of the carbon

sources, for example glucose having a concentration of at least 30 g/l. The liquid medium may also comprises maltose as one of the carbon sources, for example maltose having a concentration of at least 30 g/l. The liquid medium may further comprise at least one mineral source, for example, magnesium sulphate. In particular, magnesium sulphate having a concentration of at least 0.05 g/l. The liquid medium may further comprise monobasic potassium phosphate as a mineral source, for example, monobasic potassium phosphate having a concentration of at least 0.05 g/l as a mineral source. The liquid medium may comprise sodium nitrate at the concentration of at least 0.5 g/l. The temperature of the liquid medium in the apparatus is preferably maintained at least at 17°C.

The fungi produced by the large scale production using the apparatus according to the invention may be *Cordyceps* and/or *Ganoderma*. However, any other kind of fungi known to the skilled person and suitable for the purpose of the present invention may be produced. The fungi may be for example *Cordyceps militaris*, *Cordyceps sinensis*, *Cordyceps gracilis*, *Cordyceps memorabilis*, *Cordyceps ophioglossoides*, *Cordyceps sphecocephala*, *Cordyceps hawkesii*, *Cordyceps martialis*, *Cordyceps sobolifera*, *Cordyceps capitata*, *Ganoderma japonicum*, *Ganoderma tsugae* and/or *Ganoderma lucidum*, *Ganoderma annularis*, *Ganoderma applanatum*, *Ganoderma boniense*, *Ganoderma fornicatus*, *Ganoderma tenne*, *Ganoderma tropicum*.

The invention also provides *Cordyceps* and/or *Ganoderma* which is obtained or obtainable, using the medium of the invention, by the apparatus and/or method according to the invention,. The invention further provides *Cordyceps* paste and/or *Ganoderma* paste which is obtained or obtainable, using the medium of the invention by the apparatus and/or method according to the invention. The invention also provides *Cordyceps* and *Ganoderma* having the composition and characteristics indicated in Table 8 and Table 9, respectively.

- A standard growth medium for fungi usually comprises glucose and/or potato dextrose, malt extract and meat peptone. This standard growth medium, especially when used in a shake flask culture, yields insufficient amounts of *Cordyceps* or *Ganoderma*. It is difficult to scale up the production of *Cordyceps* or *Ganoderma* to "large scale" levels (where "large scale" level typically means using a growth medium of 400 litres or more) using the standard growth medium and without fine tuning of parameters such as stirring, aeration, pH, temperature and fermentation period.
- 10 The invention provides a growth medium different from the standard medium, which is effective in increasing the yield of the fungi, especially when used in bio-reactors where physical parameters are optimised. The medium, preferably used with the proposed production apparatus, but not limited to it, is particularly suitable for submerged fermentation providing a *Ganoderma* yield of at least 3% and a *Cordyceps* yield of at least 5%. The physical parameters comprise aeration, stirring speed, pressure, amount of anti-foaming agent, choice and amount of growth substrates and so on. It has been found that a suitable aeration rate and stirring rate in the bio-reactor can improve the growth of the mycelium (see Fig. 3 and Fig. 4). Effects of temperature and pH have been found to be less critical, as long as they are in the optimal range, e.g. of pH 4.5 to 7, and preferably pH 5.5. A positive pressure of 0.5-1.0 bar above atmospheric pressure may be applied inside the fermentation vessel to prevent contamination of the culture. Anti-foaming control may also be applied as a means of suppressing the foaming. The fermentation time of the fermentation system of the present invention is typically, but not necessarily, 48-72 hours. The actual fermentation period may last from 40 to 90 hours or longer. In comparison, fermentation time of a static/shake liquid culture is typically 15 – 30 days.
- 30 Accordingly, the invention provides a bioreactor and a multi-step process for the production of fungi comprising at least one growth medium for culturing fungi, for example *Cordyceps* and/or *Ganoderma*; the growth medium comprising at

- least glucose, maltose, and water. The growth medium may further comprise yeast extract and/or vitamins. Yeast extract comprising vitamins may be used. The growth medium may comprise yeast extract is substantially in the concentration of at least 15 g/l, in particular 15-30 g/l, more in particular 20 g/l.
- 5 The growth medium may further comprise glucose in the concentration of at least 30 g/l, in particular 30-50 g/l, more in particular 40 g/l. The growth medium may further comprise maltose in the concentration of at least 30, in particular 30-60 g/l, more in particular 40 g/l. Minerals may be, for example, sodium phosphate, monobasic sodium phosphate, dibasic sodium phosphate, potassium dihydrogen phosphate, and/or magnesium sulphate. In particular, the growth medium comprises minerals having a total concentration of at least 0.5, in particular at least 1.0 g/l. The concentration of magnesium sulphate may be of at least 0.05 g/l, in particular 0.05-1.0 g/l, more in particular 0.3 g/l. The concentration of potassium dihydrogen phosphate may be at least 0.5 g/l, in particular 0.05-2.0 g/l, more in particular 1.5 g/l. The concentration of sodium nitrate may be at least 0.5 g/l, in particular 0.5-3 g/l, more in particular 2 g/l. In particular, the growth medium according to the invention is the medium indicated in Table 1, Table 3 and/or Table 6.
- 15
- 20 The yield of *Cordyceps* using the system of the present embodiment typically averages about 5%, compared to an average of 1% using a static/shake liquid culture, giving about a 400% increase in yield. Correspondingly, the yield of bio-active components such as water soluble polysaccharides and Cordycepin (3'-deoxyadenosine, which is a nucleoside analogue), among many others, have
- 25 been found to be improved.
- Ganoderma is susceptible to impediment of growth as it tends to pelletise in a liquid growth medium (such as that recommended by US 5,334,704 which uses a potato-dextrose medium) and resulting in affected hyphal growth. In
- 30 comparison to 0.5% yield of Ganoderma by static/shake liquid culture, the yield by the new medium achieves an average of 3%, i.e. 500% better. Table 1 shows the general improvement in the yield of *Cordyceps* using the new culture

medium. The new medium shown in the table is suitable for culturing Cordyceps and Ganoderma.

- 5 The method provided by the present embodiment is a multi-stage fermentation production of Cordyceps and/or Ganoderma. Typically, a pure strain of the desired fungi is isolated (bought or somehow obtained and grown and kept on slant or plate agar). The slant culture can then be used to inoculate a first stage liquid medium culture using shake flasks. The fungi propagates under proper
10 conditions in the flasks such that its concentration high enough to seed a second stage fermentation, which comprises using a liquid medium in a larger container. Under proper conditions in the second stage, the fungi continues to grow to a concentration high enough to seed a third stage fermentation. In the present embodiment, a three-stage fermentation method is described, but
15 fermentation using more stages is also possible.

The embodiment also provides specific conditions for the growth of Cordyceps and Ganoderma in a large-scale bio-reactor, and these are described in the examples of the present embodiment given below.

20

- The medium used in the first stage, i.e. the shake flask stage, according to this embodiment, comprises a glucose concentration ranging from 30-50 g/l or more, preferably about 40g/l. It has been found that at this concentration of glucose, the yield of Cordyceps mycelium is optimised for a shake flask culture,
25 as shown in Fig. 1.

- The medium used in the intermediate and production stages, i.e. the second and third stages according to this embodiment, comprises maltose at a range of about 30 to 60 g/l, in particular 40 g/l, in the presence of minerals. It has been
30 found that at a concentration of 40g/l of maltose, if used in the presence of suitable minerals, the yield of mycelium may be optimised. Without the additional minerals, the same yield is only achievable by at least 60g/l of maltose, as shown in Fig. 2. The use of minerals in a fermentation system

therefore translates to a more productive method than one which does not comprise minerals. The minerals used in the examples are KH_2PO_4 and MgSO_4 . The type and concentration of the minerals may be different, as long as it facilitates the growth of the mycelium, for example K_2SO_4 may be used instead of MgSO_4 . The concentration of KH_2PO_4 in this embodiment may range from 0.05-2.0 g/l, in particular 1.5 g/l, and the concentration of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in this embodiment may range from 0.05-1.0 g/l, in particular 0.3 g/l. It is also preferable that there are enough vitamins in the growth medium for the production of *Ganoderma* or *Cordyceps*, and these are usually found in vitamin rich yeast extracts. It must be noted that there are some brands of yeast extracts available in the market which do not contain significant amounts of vitamins, and these are not preferred for the purpose of culturing *Ganoderma* or *Cordyceps*. Yeast extracts of 15-30 g/l, in particular 20 g/l, may be added to the medium. As a further source of carbon, the medium used in the second and third stages of the fermentation may further comprise glucose at a concentration of 35 to 50g/l, in particular 40g/l.

An example of medium according to the present invention is indicated into Table 1 and Table 6. In Table 1, the preferred values are indicated into parentheses.

For the growth of *Cordyceps militaris* and *Genoderma japonicum*, sodium nitrate may be used as the source of minerals instead of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and KH_2PO_4 , at a concentration range of 1 to 3 g/l or more, and preferably at 2 g/l. See Table 3.

Table 1. Comparison between standard and the new medium

	Shake flask		Bio-reactor			
	Cordyceps sp.	Ganoderma sp.	Cordyceps sp.		Ganoderma sp.	
	Yield g/100ml	Yield g/100ml	Yield g/100ml	PS mg/g	Yield g/100ml	PS g/100g
Standard medium (g/L)						
Glucose, 20 Bacto-peptone, 5 Malt extract, 20 Potato-dextrose broth, 24	1.24	0.31		0.2		6.6
New medium (g/L) The preferred value is indicated into parentheses						
Glucose, 30-50 (40) Maltose, 30-60 (40) KH ₂ PO ₄ , 0.05-2.0 (1.5) MgSO ₄ , 0.05-1.0 (0.3) Yeast extract, 15-30 (20)	3.22	1.66	6.96	1.5	3.0	19

Different testing methods used.

- 5 Accordingly, the invention provides a multi-stage method for cultivating fungi in a liquid medium comprising:
 - providing a source of fungi;
 - inoculating a first stage liquid medium culture using the fungi;
 - agitating the first stage liquid medium culture while letting the fungi grow
- 10 for a period of time;
 - inoculating a second stage liquid medium using the first stage culture;
 - agitating the second stage liquid medium while letting the culture grow for a period of time;
 - inoculating a third stage liquid medium of at least 500 litres contained in a
- 15 bio-reactor using the second stage culture;
 - agitating the third stage liquid medium while letting the culture grow for a period of time;

maintaining the liquid medium of each stage at a pH of at least approximately pH 5;

adding a least one carbon source to the liquid medium of each stage.

- 5 The method may further comprise steps for adding glucose, maltose, monobasic potassium phosphate, magnesium sulphate and yeast extract into the second or third stage liquid medium. The method may further comprise adding yeast extract to the second or third stage liquid medium having a concentration of at least 15 g/l. The method may further comprise the addition
- 10 of yeast extracts comprise vitamins. The method further comprises the step of adding at least one type of mineral to the second or third stage liquid medium. For instance, the mineral may comprise magnesium sulphate and/or monobasic potassium phosphate. In particular, the method comprises adding magnesium sulphate to the second or third stage liquid medium having a concentration of at
- 15 least 0.05 g/l. The method further comprises the step of adding monobasic potassium phosphate to the second or third stage liquid medium such that it has a concentration of at least approximately 0.05 g/l.

In particular, the method of the invention further comprises:

- 20 culturing the fungi in first stage liquid medium for a period of at least 48 hours; wherein
- the liquid medium has a volume of at least approximately 500 ml.

- The step of agitating the first stage liquid medium may comprise the step of
- 25 rotating the container of the medium at a rate of at least 100 rpm and/or comprising: aerating the second stage liquid medium at a rate of at least 1:0.5 v/v/m; and culturing the fungi in a volume of at least approximately 50 ml of the second stage medium for at least 40 hours. The step of agitating the second stage liquid medium may comprise stirring the second stage liquid medium at a
- 30 speed of at least 100 rpm and/or aerating the third liquid medium at a rate of at least 1:0.5 v/v/min; and culturing the fungi in a volume of at least approximately 500 litres of the third stage medium for at least 48 hours.

The method of any embodiment of the invention may further comprise the step of maintaining a temperature of at least 17°C in the liquid medium. In particular, in the method of the invention if the fungus is *Cordyceps militaris*; and the method further comprises the step of maintaining the temperature of the liquid medium at least 17°C. In fact, it has been found that *Cordyceps militaris* does not grow at a temperature higher than 25°C (see Fig.10). Fig.10 shows that the growth of *Cordyceps militaris* in slant/plate agar in presence of YM medium (see Table 2) decreases at 25°C. No growth was observed at 29°C.

In the method of the invention, the culture of *Cordyceps militaris* comprises the step of growing the *Cordyceps militaris* in a medium comprising sodium nitrate. In particular, the concentration of sodium nitrate in the medium is at least 0.5%.

In the method of the invention, the medium may comprise a concentration of Riboflavin of at least 0.01%.

According to a further aspect, the invention provides a Ganoderma and/or Cordyceps paste prepared by growing Ganoderma and/or Cordyceps mycelium according to the invention and further comprising the steps of collecting the mycelium and filtering it. Any known filter and filtering system suitable for the preparation of the paste according to the invention may be used. For example, through filtering cloth by gravity or by adjustable or controlled vacuum filters such as rotary drum vacuum filters. The obtained wet mycelium is collected (it generally contains 10-15% of total solids, namely 85-90% of moisture) and then washed (preferably twice) to remove the residues of the fermentation medium. The Ganoderma and/or Cordyceps paste can also be made from Cordyceps and/or Ganoderma spores as described in the examples. The Ganoderma and/or Cordyceps paste prepared according to any embodiment of the invention can be used for the preparation of "fortified" beverages and/or food. For example, for the preparation of fortified drinks, and dairy products. In particular, the Cordyceps paste comprises components (on dry wt. basis) of: total protein:

27-28%; total water soluble polysaccharides: at least 20%; Cordycepic acid (mannitol): at least 5%; Cordycepin (3'-deoxyadenosine): at least 0.1%; adenosine: at least 0.15%. The product specification of Cordyceps in paste form is shown in Table 13. The product specification of Ganoderma in paste form is shown in Table 14.

The spores of Cordyceps and Ganoderma species are classified as the genesis of Ascospores and Basidiospores, respectively. Ascospores are delimited inside the ascus by free cell formation, that is, spore boundaries are demarcated within the mass of cytoplasm and out of contact with the ascus plasmalemma, while the basidiospore is basically very uniform, viz. a generally ellipsoidal cell produced at the apex of a tapering sterigma.

In general, the spore of medicinal fungi have special or unique therapeutic properties different from its fruiting body or mycelia, though the medical properties of Cordyceps spores are not completely known. Spores differ from fruiting body in that active components of spores are more concentrated in the spores than in the fruiting body. Accordingly, the efficacy of spores is higher than that of the fruiting body. In fact, in the case of spores of Ganoderma lucidum, effects of spores are 75 times higher or stronger than that of fruit body.

For Ganoderma, the commercial production of spores are based on the farming technology. For example, in the commercial production of Ganoderma lucidum (popular name: Lingzhi) spores, the spores, "stationed" on the umbrella shape of the fruiting body, are collected by a farming technology when mature.

As regards to the Cordyceps species, no methods for technology to produce the spores are available in the prior art. For example, for wild grown or farm cultivated Cordyceps sinensis (popular name: Dong Chong Xia Cao or caterpillar fungus), a special fruiting body, a stroma, is formed. However, no scientific study or literature has ever indicated where the spores of the Cordyceps sinensis are "stationed" and can be collected from.

The present inventors have developed a method for the commercial production of spores of Ganoderma and/or Cordyceps species by liquid and submerged cultivation system.

5

As mentioned above, the production of Ganoderma spores is dependent on the fruiting body generated by the farming process. With reference to Cordyceps, no production of spores has been described in the prior art. The present invention provides for the first time a method for the commercial preparation of spores of Cordyceps. According to the method of the prior art, about 1000 kg of dry fruiting body of Ganoderma lucidum can only produce 1 kg of dry spores, that is a yield of about 0.1%. By using the method according to the invention, 30 kg of dry mycelia can produce 2-3 kg of dry spores of Ganoderma as well as of Cordyceps, with a yield of about 6-10%.

15

Accordingly, the invention provides Ganoderma spores and/or Cordyceps spores and processes for their preparation. The medical properties of Ganoderma and/or Cordyceps spores are mentioned below. Besides being used as health supplements or medicine, spores can also be used for food fortification and cosmetic application. For example, such spores may be useful for the preparation of Ganoderma and/or Cordyceps paste and also for the preparation of fortified beverage or food according to the invention.

The present invention further provides a food and beverage comprising the Cordyceps and/or Ganoderma mycelium and spores, and the Cordyceps and/or Ganoderma paste prepared according to any embodiment of the present invention. The beverage mentioned above may be: yogurt, liquid yogurt, juice, milk, soy milk, alcohol (alcoholic drinks), coffee, black tea, green tea, oolong tea, sport drinks and the like. The food mentioned above may be: baked confection such as cookies, bread, cakes, rice crackers and the like; cold confection such as pudding, jelly, ice-cream and the like; sweets such as chewing gum, candies and the like; snacks such as crackers, chips and the like;

noodles and the like; pate-like food made from fish or meat (fish cake), ham and fish sausages and the like; seasonings such as miso, soy sauce, dressings, mayonnaise, and sweetening and the like; and various dishes such as tofu, konnyaku, Chinese dumplings, crockets, salad, soup, stews and the like. The beverage and food may be prepared according to known and standard techniques by mixing at least one of Cordyceps and/or Ganoderma mycelium and spores, or the Cordyceps and/or Ganoderma paste prepared according to any embodiment of the present invention with the suitable food and/or drink ingredients.

10

In particular, the present invention provides a "fortified" milk comprising mixing milk, for example, any kind of liquid milk, such as whole milk, skim milk, reconstituted milk, and the like, with at least one of Ganoderma mycelium, Cordyceps micelyum, Ganoderma spores, Cordyceps spores, Ganoderma paste and Cordyceps paste produced in the presence of the medium of the invention. More in particular, the fortified milk prepared by mixing milk and Ganoderma paste and/or Cordyceps paste of the invention maintains the integrity of the components of Ganoderma and/or Cordyceps, the physical properties of the Ganoderma and/or Cordyceps paste, particularly in solubility and dispersibility, integrity of milk components as well as integrity of milk flavour and natural colour. The analysis of the Cordyceps fortified milk is given in Table 16.

20

In another aspect, the invention provides a pharmaceutical composition comprising at least one of Ganoderma mycelium, Cordyceps micelyum, Ganoderma spores, Cordyceps spores, Ganoderma paste and/or Cordyceps paste, produced in presence of the medium of the invention. Optionally, the pharmaceutical composition comprises at least a pharmaceutically acceptable eccipient and/or diluent.

25

30

The invention also provides the use of Ganoderma mycelium, Cordyceps micelyum, Ganoderma spores, Cordyceps spores, Ganoderma paste and/or

Cordyceps paste, produced in presence of the medium of the invention, for the manufacture of a pharmaceutical composition, beverage (for example milk) and/or food (for example, nutritional supplement) for the use in therapy.

5 Medical authorities have allowed the Cordyceps and Ganoderma for the following uses:

- Support of immunological system, kidney and respiratory function;
- Support in maintaining healthy blood glucose and cholesterol level;
- 10 • Increase vitality;
- Enhance circulatory function in the body

The following pharmacological effects of Ganoderma and Cordyceps which have been studied by either animal models and/or human (clinical) trials:

15

- IMMUNOMODULATION: Cordyceps sinensis (CS) can significantly enhance the immunity of the body.
- ANTI-CANCER AND ANTI-MICROBIAL EFFECTS: Associated with immunomodulatory function, CS shows an anti-cancer effect, and was reported to inhibit viral, bacterial and fungal infections.
- 20 • ANTIDIABETIC ACTIVITY: Polysaccharide in CS was reported to significantly lower blood glucose levels.
- ENERGY METABOLISM AND PERFORMANCE ENHANCEMENT: CS was reported to stimulate hepatic energy metabolism.
- 25 • RESPIRATORY AND PULMONARY FUNCTIONS: CS was reported to relax the bronchial walls as well as having sedative, antitussive, and anti-inflammatory properties, thus it is effective against cough, phlegm, asthma and other respiratory ailments.
- CHOLESTEROL AND LIPID METABOLISM: The report indicated plasma triglyceride, "bad cholesterol" such as LDL-c and vLDL-c, and total
- 30 cholesterol levels were lowered by the CS.

- EFFECTIVE ON RENAL DISORDER: There are indications that CS may be of use in the treatment of Berger's disease (IgA nephropathy).
- EFFECTIVE ON SEXUAL DYSFUNCTIONS AND REPRODUCTIVE DISORDER: Clinical improvement of impotence by taking CS was reported.
- 5 • OTHERS: The reports suggested that CS can stimulate erythropoiesis in mouse bone marrow, and inhibits platelet aggregation.

With particular reference to the strain of *Ganoderma lucidum* and *Cordyceps sinensis* the following medical effects have been described:

10

1) Medical effects of *Ganoderma lucidum* fruit body & spores:

- ANTI-CANCER AGENT: cancer studies in animals have shown a 50% tumour regression rate with *Ganoderma lucidum* (popular name: Lingzhi) treatment
15 (e.g., connective tissue cancer model in mice).
- IMMUNE SYSTEM ENHANCEMENT (Bronchitis, Asthma, Allergies, Herpetic Conditions and HIV Infection): chronic bronchitis in the elderly has been shown to respond favourably to treatment using a concentrated Lingzhi mushroom product in a trial involving 2,000 cases in China. This study
20 demonstrated a better than 60% success rate.
- ANTI-VIRAL ACTIVITY: a specific protein-bound polysaccharide component of Lingzhi extract, known as GLhw-02, has been shown to possess potent anti-viral properties against herpes simplex virus type-1 and type-2 under experimental conditions. Under experimental conditions, various ganoderic
25 acids in Lingzhi mushroom extract have been shown to be active anti-HIV agents, showing an ability to reduce viral replication by 50% at conservative doses.
- CARDIOVASCULAR HEALTH (High Blood Pressure and Reduced Platelet Aggregation): two human controlled studies revealed that Lingzhi extract can
30 reduce high blood pressure to a significant degree (systolic and diastolic),

even in patients who had previously failed to respond to established anti-hypertensive medications.

- LIVER PROTECTIVE EFFECTS (Hepatoprotective Properties): Lingzhi is prescribed in China for the treatment of chronic and acute hepatitis. Various ganoderic acids in Lingzhi mushrooms have strong antihepatotoxic properties, which under experimental conditions have been shown to protect liver cells from chemically-induced injury, including protection from the highly toxic and lethal substance, carbon tetrachloride.

2) Medical effects of Cordyceps fruit body, mycelia & spores:

- IMMUNOMODULATION: Cordyceps sinensis (CS) can significantly enhance the immunity of the body.
- ANTI-CANCER AND ANTI-MICROBIAL EFFECTS: associated with immunomodulatory function, CS shows an anti-cancer effect, and was reported to inhibit viral, bacterial and fungal infections.
- ANTIDIABETIC ACTIVITY: polysaccharide in CS was reported to significantly lower blood glucose levels.
- ENERGY METABOLISM AND PERFORMANCE ENHANCEMENT: CS was reported to stimulate hepatic energy metabolism.
- RESPIRATORY AND PULMONARY FUNCTIONS: CS was reported to relax the bronchial walls as well as having sedative, antitussive, and anti-inflammatory properties, thus it is effective against cough, phlegm, asthma and other respiratory ailments.
- CHOLESTEROL AND LIPID METABOLISM: the report indicated plasma triglyceride, "bad cholesterol" such as LDL-c and vLDL-c, and total cholesterol levels were lowered by the CS.
- EFFECTIVE ON RENAL DISORDER: There are indications that CS may be of use in the treatment of Berger's disease (IgA nephropathy).
- EFFECTIVE ON SEXUAL DYSFUNCTIONS AND REPRODUCTIVE DISORDER: clinical improvement of impotence by taking CS was reported.

- OTHERS: the reports suggested that CS can stimulate erythropoiesis in mouse bone marrow, and inhibits platelet aggregation.

(Wang and Shiao, *Journal of Food and Drug Analysis*, Vol.8, No. 4, 248-257, 2000; Institute for Natural Products Research, http://www.naturalproducts.org/inpr/monographs_pdf/index.html; Anon. *Great Pharmacopoeia of Chinese Materia Medica* (Chinese). 1997, Shanghai Publishing House of Science & Technology, Shanghai, China; Ming-Shang MIAO, *Dieto-therapy: Pharmacology of Chinese Materia Medica* (Chinese). 2001, Scientific Publishing House, Beijing, China)

Accordingly, the invention also provides a method for therapeutic treatment, in particular (but not limited to) for the treatment of tumors, stimulation of macrophages activity, or stimulation of immunological system, stimulation of kidney and respiratory functions, enhance the circulatory function of the body, and other uses as mentioned above, comprising the administration of at least Ganoderma mycelium, Cordyceps micelyum, Ganoderma spores, Cordyceps spores, Ganoderma paste and/or Cordyceps paste, produced in presence of the medium of the invention, or a pharmaceutical composition, beverage (for example milk) and/or food (for example, nutritional supplement) comprising at least Ganoderma mycelium, Cordyceps micelyum, Ganoderma spores, Cordyceps spores, Ganoderma paste and/or Cordyceps paste, produced in presence of the medium of the invention.

Examples are given below of the production of various strains of Cordyceps and Ganoderma. It should be noted that the parameters given for the conditions of growth such as fermentation period, temperature, stirring speed, aeration rate, pH rage etc, are not necessarily limited to the values as shown but may vary within a reasonable degree as is typically understood by people skilled in the art.

Example 1: Cultivation of *Ganoderma japonicum* in fermentation device

This example used a liquid culture medium comprising at least one carbon source, yeast extracts, vitamins (the yeast extract usually, though not always, contains enough active vitamins without the need for an additional supplement of vitamins), minerals and other growth factors such as sucrose, glucose, peptones and food grade chemicals. It has been found that yeast extracts, which are vitamin-rich, are particularly encouraging to the growth of *Ganoderma*.

In order to produce *Ganoderma japonicum* APN 3-1 (FIRDI-36445) the spores of a suitable strain, such as *Ganoderma lucidum* APN 3-1 (FIRDI-37177) were cultured on and kept in flowing slant or plate agar medium (Table 2) at 25 - 30°C (preferably 29°C) for 3-5 days. A suitable amount of fungi was then harvested and used to inoculate a liquid growth medium in shake flasks to make a first seeding culture.

A typical example of a shake culture was prepared comprising a liquid growth medium in Erlenmeyer flasks, and 1 litre of broth medium (Table 3) in a 5 litre conical flask, autoclaved at 121°C for 20 minutes, and inoculated with 6 of the slant cultures of *Ganoderma lucidum* APN3-1, and subjected to the following growth conditions:

- pH of 5.5 (adjusted before inoculation)
- 29°C
- the flasks rotated on shakers at a speed of about 160rpm
- culture period of 72 hr.

The 1st seeding culture was terminated when the growth rate reached the stationary phase. A suitable amount of cells from the 1st culture was then used for the 2nd seeding culture in a liquid medium in small stirred tanks.

The 2nd seed culture used about 50 litres or more of the fermentation medium (Table 1) in 75 litre bio-reactors, for example, a bio-reactor of the brand BioLafitte or bigger (for example comprising 800 litres or more). The systems
 5 were sterilised at 121°C for 30 minutes and inoculated with 2 litres of the 1st seed culture (or about 4% of inoculum, i.e. 2 litres to 50 litres). The following growth conditions were maintained:

- pH 5.5 (adjusted before inoculation)
- 10 • 25°C
- aeration rate 1:0.5v/v/m to 1:0.9v/v/m (i.e. 1500-2700 litres/hr for 600 litre liquid medium) where "v/v/m" means culture medium volume/aeration flowrate.
- turbo stirrers speed 200 rpm.
- 15 • cultivation period 48 hours

For the large scale production of mycelia of *G. lucidum*, about 50 litres or more of the 2nd seeding culture, or 8% of inoculum, was used to inoculate 800 litres stirred tank reactors (BioLafitte) or bigger containing typically 600 litres or more
 20 of a suitable fermentation medium. The volume of the medium may actually range from at least 400 litres and above. The tanks containing the medium are first sterilised at 121°C for 30 min and were maintained under the following conditions:

- 25 • pH 5.5 (adjusted before inoculation)
- 25°C.
- stirring speed 200 rpm.
- aeration rate 1:1 v/v/m (about 36.0 m³/hr for 600 litre).
- cultivation period 72 hr

30

A yield of at least 3% for *G. japonicum* can be obtained.

The mycelium was collected by drawing off the culture from the reactors and filtering it using a vacuum filter, a filter press or hydrostatic press. Up to 60-100 kg of mycelium cake with 30% dry matter was obtained per reactor. This cake was then dried on a double-cone rotary vacuum dryer or by freeze-drying and filamentous flour with 28-30% of protein was obtained. The filamentous flour was grounded by a miller to a particle size of 100-200 meshes. The obtained powder was then packaged into aluminium bags.

10 **Example 2: Cultivation of *Ganoderma lucidum* and *Ganoderma tsugae* in a fermentation device**

G. lucidum APN 3-2 (FIRDI-37177) or G. tsugae APN 3-3 (FIRDI-36090), was firstly grown on and kept in the slant or plate agar medium (Table 2) at 24°C for 3-5 days. The slant cultures of G. lucidum or G. tsugae were then used to inoculate 50 ml of liquid growth medium (Table 3) in a 250-ml Erlenmeyer flask. The mycelia were subjected to shake culture at 25 - 29°C (preferably 27°C) for 72 hr on a rotating shaker at 160 rpm.

20 For commercial production of G. lucidum and G. tsugae, multi-stage fermentation as mentioned in Example 1 was used. The mycelia yields of G. japonicum and G. tsugae are close to that of G. lucidum.

Table 2: YM slant/plate agar medium

Ingredient	Content
Yeast extract	3g
Malt extract	3g
Peptone	5g
Dextrose	10g
Agar	20g
Distilled water	Up to 1 litre

Table 3 Broth Medium for *Cordyceps militaris* and *Ganoderma japonicum*

Ingredients	Content, g/l
Glucose	40g
Maltose powder	40g
NaNO ₃	2g
Yeast extract/peptone	20g
Water	Up to 1 litre

The culture medium (Table 1) for the cultivation for all other strains of *Ganoderma*, however, differs slightly from that used specifically for *Ganoderma japonicum* (Table 3). For all other strains of *Ganoderma*, KH₂PO₄ and MgSO₄·7H₂O are used as the mineral source, but for *Ganoderma japonicum* the culture medium having composition as in Table 3 is preferably used (wherein the mineral is NaNO₃) .

10 **Example 3: Cultivation of *Cordyceps sinensis* in fermentation device**

A liquid culture medium comprising a carbon source, a nitrogen source, vitamins, minerals and other growth factors from raw materials of glucose, maltose or powder, yeast extract, and food grade chemicals was used. In order to produce the *Cordyceps*, for example, *C. sinensis*, an isolated strain was firstly subjected to solid medium culture. The strain *C. sinensis* APN 4-1 (FIRDI-36421), was grown on and/or kept on YM slant or plate agar medium (Table 2) or the PDA medium (Table 4) in test tubes or agar plates at 25°C for 3 days.

For the 1st seed culture, shake culture making use of Erlenmeyer flasks was used as described in Example 1. One litre of the broth medium (Table 5, or Table 6) contained in each 5 litre conical flask was sterilised at 121°C for 20 minutes and inoculated with 3-6 slant or plate culture of the *C. sinensis* APN 4-1 and were then grown under the following conditions:

- pH 5.5 (adjusted before inoculation)
- 25°C

- the flasks rotated on shakers at a speed of about 150rpm
- culture period of 48 hr.

5 For the 2nd seed culture of *C. sinensis*, 75 litre (or bigger) stirred bio-reactors (BioLafitte) each containing 50 litres or more of the fermentation medium (Table 1) were sterilised at 121°C for 30 minutes and then inoculated with 2 litres of the cells from the 1st seed culture (4% of inoculum). The following growth conditions were maintained:

- 10
- pH 5.5
 - 25°C
 - aeration rate of 1:0.2-1:0.9 v/v/m (1500- 2700 l/hr for 50 litres of medium)
 - stirring speed 200rpm
- 15
- cultivation period 48 hr

20 For the cultivation of mycelia of *C. sinensis*, 800 litre (or a bigger) stirred-tank reactors (for example, BioLafitte), each containing a volume of 600 litres or more of the fermentation medium (Table 1) are inoculated with 50 litres or more of 2nd seeding culture (8% of inoculum). The tanks and the fermentation medium were sterilised at 121°C for 30 minutes before inoculation.

- 25
- pH 5.5
 - 25-29°C
 - stirring speed 200 rpm
 - typical aeration rate of 1:0.5 -1:1 v/v/m (18-36.0 m³/hr for 600 litre medium)
 - cultivation period 48-72 hr

30 When the mycelium had the end of its exponential phase or had multiplied enough for the purpose of production, the bottom valves of the 800 litre bio-

reactors were opened, and the mycelium of *C. sinensis* was collected and filtered on vacuum filter or filter pressed; up to 100 kg of mycelium cake with 30% of dry matter was obtained from each bio-reactor. This cake was then dried on a double-cone rotary vacuum dryer or by freeze- drying and a filamentous flour with 27-28% of protein was obtained. The filamentous flour was grounded by a miller to a particle size of 100 -200 mesh. The obtained powder was then packaged into aluminium bags.

Table 4. Potato dextrose agar medium

Ingredients	Content
Potato extract	4.0g
Glucose	20.0g
Agar	15.0g
PH	5.6
Distilled water	Up to 1 litre

Table 5. Flask medium-I

Ingredients	Content g/l
Glucose	40.0
Maltose powder	20.0g
Potato dextrose broth	24.0g
Yeast extract	10.0
Water	Up to 1 litre

Table 6. Flask medium-2

Ingredients	Content g/l
Glucose	40.0
Maltose powder	40.0
KH ₂ PO ₄	1.5
MgSO ₄ .7H ₂ O	0.3
Yeast extract/peptone	20.0
Water	Up to 1 litre

Example 4: Cultivation of *Cordyceps gracilis*, *Cordyceps memorabillis* and *Cordyceps ophioglossoides* in fermentation device

The strains *Cordyceps gracilis* APN 4-2 (FIRDI 32217), *Cordyceps memorabillis* APN 4-3 (FIRDI 32218) and *Cordyceps ophioglossoides* , were grown and kept on YM slant or plate agar medium (Table 2) at 25°C for 3-5 days. The slant cultures of *C. gracilis*, *C. memorabillis* or *C. ophioglossoides* were used to inoculate 50 ml of growth medium (described in Table 5, 6) in 250-ml Erlenmeyer flasks. The mycelia were subjected to shake culture at 25° C for 72 hr on a rotating shaker at 150 rpm.

For commercial production of *C. gracilis*, *C. memorabillis* and *C. ophioglossoides*, the multi-stage fermentation as described in Example 3 was used.

15

Example 5: *Cordyceps militaris*

It was found that this strain would not grow well at a temperature higher than 25°C and also would not grow well on the agar slant/plate. By addition of 0.6% NaNO₃ or 0.01% Riboflavin (vitamin B2) to the YM agar medium (control, Table 2), the cultivation time was shortened to 3 days (grown on the fortified medium) from 7 days. *Cordyceps militaris* APN 4-5 (FIRDI 32219) was grown and kept on fortified YM medium at 17-23°C, preferably at 20°C for 3-days before using it for inoculation of the multi-stage fermentation in Example 3.

25

The culture medium (Table 1) for the cultivation for all other strains of *Cordyceps*, therefore, differs slightly from that used specifically for *Cordyceps militaris* (Table 3). For all other strains of *Cordyceps*, KH₂PO₄ and MgSO₄·7H₂O were used as the mineral source, and for *Cordyceps militaris* NaNO₃ (Table 3) was used instead.

30

The methods of extraction of the active ingredients in Cordyceps or Ganoderma presently used in the prior art, do not include any step of hot water extraction. The invention provides a method which includes a step of hot water extraction which increases the amount of active components recoverable from the medicinal fungi. The water used may also be mixed with a pH buffer, adjusting it to about pH 10.

Example 6: Extraction process of Cordyceps bio-active ingredients

According to conventional methods known in the art, the active ingredients of Cordyceps or Ganoderma are extracted for water-soluble and ethanol-soluble compounds. However, these conventional methods of extraction do not include hot water extraction, which is provided in this embodiment and is described below. The absence of a hot water extraction step in conventional methods makes them ineffective in extracting the water-soluble polysaccharides. The hot water may also be buffered at pH 10 for further efficacy.

About 1700g of wet Cordyceps mycelia were soaked, with gentle agitation, with about 6 litres of hot water (about 90°C) overnight, i.e. about 24hrs or more. The Cordyceps were then filtered and the filtrate was then mixed with 2 volumes of 99% ethanol (at 6 litres per volume). The precipitate resulting from the addition of the ethanol to the filtrate was then collected and washed in 50% ethanol, and subsequently lyophilised, obtaining 2.9g of Cordyceps polysaccharides.

The residue was then extracted for active compounds for a further 4 times by using about 4 litres of 80% ethanol solution, with gentle agitation. The ethanol was then evaporated and the resultant concentrate was freeze-dried, obtaining 101g of solids. The use of any other solvent other than methanol, acetone, will be evident to any skilled in the art.

Both the water extract and the ethanol extract can be used for therapeutic treatments.

Example 7: Sporulation of Ganoderma.

After 48-72 hours of cultivation of Ganoderma in a bio-reactor according to the examples 1-5, wherein the pressure was raised to at least 2 bar, and aeration rate adjusted to 1:0.4 to 1:0.6 v/v/m (about 1350-1500 litres/hr), 0.1 g of Ganoderma spores per litre of broth were produced. At such a pressure, it has been found that Ganoderma produces basidiospores as shown in Fig. 5.

10 The mycelium was collected by drawing the culture from the reactors and filtered by using a vacuum filter or a filter press to obtain the mycelium cake with 30% dry matter. Alternatively, it can be filtered through a needle-felt polyester filter medium by gravity or by a controlled vacuum force to make the wet mycelium containing 10-20% of total solids.

15

The spores of Ganoderma were passed through the filters and remained in the filtered fermentation broth (liquid residues). A range of spore counts from 10^6 – 10^7 per ml of the filtered fermentation broth was obtained for different species of Ganoderma. The filtered fermentation broth was subjected to centrifugation by either a nozzle-discharge centrifuge to concentrate the broth to 30-40% of total solids or by with twice-washing until the fermentation residues have been removed. The concentrated solution of the spore was then dried by spray drying or freeze-drying. A yield of up to 3.0 gram per liter fermentation broth was obtained.

25

Prior to this invention, such spores of Ganoderma could only be produced on solid growth medium and liquid growth medium was mainly used to produce mycelia. These spores of *Ganoderma* are considered to be much more therapeutic as compared to the fruiting bodies in TCM, and are highly valuable.

30

Example 8: Sporulation of Cordyceps

Cordyceps spores were produced in the same way as described for Ganoderma in Example 7.

5

Example 9: Variable conditions of Examples 1-8

The parameters in the examples given above may be adjusted as it may appear evident to any skilled in the art. For example, the fungi of Ganoderma and
10 Cordyceps grow at a temperature in the range of 25 to 30°C, and at a pH range of pH 4.5 to pH 7.0, and may be cultivated depending on the parameters for periods ranging from 40 hours to 90 hours. These parameters apply to any stage of the fermentation, although they may be optimised at a different value at each stage.

15

However, some strains of Cordyceps, such as *Cordyceps militaris*, thrive in a temperature range of 17° to 23°C, and preferably at 25°C. Therefore the minimum temperature of such a fungi production system is preferably 17°C.

20 The aeration rate is usually at least 1:0.5 v/v/m to the maximum the bio-reactor container can reasonably sustain for a given volume of the culture medium. Similarly, the stirring speed or the speed of rotating the shake culture flasks may be at least 100 rpm to any rate which the containers can sustain for a given volume of culture medium.

25

The volumes of the first stage fermentation is usually 1 litre in a 5 litre conical flask, but it may be as low as 500ml. A second stage fermentation container usually has a volume of growth medium of 50 litres in a typical container of 75 litres. However, in order to be productive a volume of at least 30 litres may be
30 sufficient. The third stage, or production stage, of the fermentation may be at least 400 litres although it is typically 600 litres or more.

The composition of the novel medium given in Table 1 shows the preferable concentrations of the components in the medium. However, it is possible to cultivate the fungi with at least 30 g/l of glucose, 30 g/l of maltose, 15 g/l of yeast extract, 0.05 g/l of KH_2PO_4 and 0.05 g/l of MgSO_4 .

5

For the growth medium of *Cordyceps militaris* and *Ganoderma japonicum*, the source of mineral used is NaNO_3 instead of KH_2PO_4 and MgSO_4 . It is possible to sustain a reasonable growth rate at a concentration of 1 g/l although the preferable concentration is 2 g/l.

10

Table 7, Table 8 and Table 9 below show the difference in quality between that of wild *Cordyceps* and *Ganoderma* powders and that of *Cordyceps* and *Ganoderma* grown by the proposed embodiments.

15 Chemical and hygienic tests of *Cordyceps* and *Ganoderma* powders on the market

Table 7. Analysis

Items	<i>Cordyceps</i>	<i>Ganoderma</i>
10% pH	5.5-6.0	5.5-6.5
Moisture %	4	4
Protein (Kjeldhal, N x 6.25)	28.9	27.7
Ash (Muffle furnace, 550°C)	6.60	4.73
Copper, Cu (ppm)	21	7.8
Arsenic, As (ppm)	<1	<1
Lead, Pb (ppm)	<1	<1
Mercury, Hg (ppm)	<0.1	<0.1
Total aerobic plate count, cfu/g	500	260
Total mould & yeast, cfu/g	200	100
<i>E. coli</i> , <i>Salmonella</i> spp, and <i>Staphylococcus aureus</i>	Absent	Absent

20

Table 8. Product specifications of powdered *Cordyceps* obtained by the method provided in the embodiment

Product description	Powdered <i>Cordyceps</i> mycelia, produced by submerged cultivation, followed by drying, grinding And sterilization
Product fingerprints Proteins/glycoproteins: Overall:	SDS-PAGE and western blotting Fourier transform infrared (FTIR)spectroscopy
Physical appearance: Colour: Odour: Particle size: Flowing property:	Powder with good flow property Brown or light brown Slightly fermented 100 to 200 mesh, Free-flowing
pH (in 10% solution):	4.5 – 5.5
Moisture %:	Less than 5 %
Active components: Total protein: Total water soluble polysaccharides: Cordycepic acid (mannitol): Cordycepin (3'-deoxyadenosine): Adenosine:	27- 28% 20% or above 5% or above 0.1% or above 0.15% or above
Ash:	Less than 5%
Heavy metal : Arsenic Copper Lead Mercury	< 0.01 ppm < 15 ppm < 0.2 ppm < 0.01 ppm
Microbial limit: Total bacterial count, CFU/g Total yeast & mould, CFU/g Total E. coli, per gram Staphylococcus aureus, per gram Salmonella spp., per gram	In compliance with the Singapore Medicines Act < 10 ² < 10 ² Absent Absent Absent

Table 9: Product specifications of powdered *Ganoderma* obtained by the method proposed in the embodiment

Product description	<i>Ganoderma</i> mycelia cultivated by submerged method, followed by drying of mycelium, and grinding to powder.
Product fingerprints Proteins/glycoproteins: Overall:	SDS-PAGE and western blotting Fourier transform infrared (FTIR)spectroscopy
Physical appearance: Colour: Odour: Particle size: Flowing property	Powder with good flow property Light yellow or beige Slightly fermented 100 to 200 mesh, Free-flowing
pH (in 10% solution):	5.5 – 6.5
Moisture %:	Less than 5 %
Total protein:	28-30%
Total water soluble polysaccharides (PS): (grams / 100gram powder)	More than 15%
Ash:	Less than 7%
Heavy metal: Arsenic Copper Lead Mercury	In compliance with the Singapore Medicines Act < 0.01 ppm < 15 ppm < 0.2 ppm < 0.01 ppm
Microbial limit: Total bacterial count, CFU/g Total yeast & mould, CFU/g Total E. coli, per gram Staphylococcus aureus, per gram Salmonella spp., per gram	In compliance with the Singapore Medicines Act < 10 ² < 10 ² Absent Absent Absent

- 5 The fungi produced by the embodiments of the inventions may be used in compositions which comprise mixtures of several *Cordyceps* and *Ganoderma* strains. Three examples (formulas 1-3) are given below in Tables 10 - 12:

Table 10. Formula one (CordyDouble)

	Dosage, per capsule
Cordyceps sinensis mycelia	175 mg or 350 mg
Cordyceps militaris mycelia	75 mg or 150 mg

Table 11. Formula two (GanoVersicolor Plus)

	Dosage, per capsule
Ganoderma japonicum mycelia	100 mg or 200 mg
Trametes versicolor mycelia	100 mg or 200 mg
Ganoderma lucidum spores	50 mg or 100 mg

5

Table 12. Formula three (Ultra-Booster)

	Dosage, per capsule
Cordyceps sinensis mycelia	100 mg
Cordyceps militaris mycelia	50 mg
Cordyceps gracilis mycelia	50 mg
Cordyceps memorabilis mycelia	100 mg
Cordyceps ophioglossoides mycelia	50 mg
Ganoderma lucidum spores	100 mg
Ganoderma japonicum mycelia	50 mg

10 **Example 10: Preparation of Paste Form of Cordyceps**

Upon completion of the large-scale cultivation methods described in Example 1-5, the mycelium, instead of being collected and dried, was collected by filtering it through a needle-felt polyester filter medium by gravity or by a controlled vacuum force to make the wet mycelium contain 10-20% of total solids. The wet mycelium was then washed twice by water to remove the residues of the medium.

The yield of the Cordyceps paste obtained ranged 250-500 kg per cubic meter of fermentation broth.

20

The wet mycelium with 10-20% of total solids was subjected to a homogenizing process by either a colloidal mill, or by a homogeniser, or by any type of proper milling machine. A particle size of 0.5-3.0 μm of the wet mycelium was obtained by homogenization. This particle size allowed the wet mycelium to be well dispersed in the solutions. The obtained Cordyceps preparation is indicated as "paste Cordyceps".

The specific characteristics of the obtained "paste Cordyceps sinensis" are as shown in Table 13. A picture of mycelia and spores of Cordyceps sinensis is shown in Figures 8 and 9.

Table 13: Product specification of *Cordyceps sinensis* in paste form

Product description	<i>Cordyceps sinensis</i> mycelia produced by submerged cultivation, followed by filtration, packaging and retorting at 110 -121°C.
Packaging form	Paste form of products, quantity in 3 kg or customised size, packed in laminated retort pouch.
Product fingerprints Proteins/glycoproteins: Overall:	▪ SDS-PAGE and western blotting ▪ Fourier transform infrared (FTIR) spectroscopy
Physical appearance: Colour: Odour: Property:	Brown or light brown Slightly fermented Paste
pH (in 10% solution):	4.5-5.5
Total solid %:	10-20 %
Components (dry basis): Total protein: Total water soluble polysaccharides: Cordycepic acid (mannitol): Cordycepin (3'-deoxyadenosine): Adenosine:	27 - 28% 20% or above 5% or above 0.1% or above 0.15% or above
Ash:	Less than 5%
Heavy metal: Arsenic	In compliance with the Singapore Medicines Act

Copper Lead Mercury	< 0.01 ppm < 15 ppm < 0.2 ppm < 0.01 ppm
Microbial limit: Total bacterial count, CFU/g Total yeast & mould, CFU/g Total E. coli, per gram Staphylococcus aureus, per gram Salmonella spp., per gram	In compliance with the Singapore Medicines Act < 10 ² < 10 ² Absent Absent Absent

Different characteristics of other species or strains, other than *Cordyceps sinensis* (*C. sinensis*) have also been observed. The characteristics are shown in Table 14.

5

Table 14: Characteristics of other *Cordyceps* strains

Product and cell description	1) <i>C. militaris</i> shows the whitest and finest mycelia visually, while <i>C. sinensis</i> is the most coarse. 2) The paste of <i>C. militaris</i> is more like milk flavour 3) The taste of powdered <i>C. militaris</i> is the sweetest, that of <i>C. ophioglossoides</i> a little sour, and the bitter taste of <i>C. sinensis</i>. 4) Filtered bio-mass of <i>C. militaris</i> mycelia is more compact than that of <i>C. sinensis</i> & <i>ophioglossoides</i>. 5) Under microscopy, two spores are contained inside the ascus of <i>C. militaris</i>, as comparison with that of <i>C. sinensis</i>, only one spore (see photos)
FTIR fingerprints	Fingerprints of five species of <i>Cordyceps</i> in Fourier transform infrared (FTIR) spectroscopy are very different, which can be provided if required.
Active compound: Cordycepic acid	Contents of <i>C. militaris</i> & <i>C. ophioglossoides</i> are higher than 9%, while <i>C. sinensis</i> is 5-7% only.
Active compound: Cordycepin	The content of <i>C. militaris</i> is double or triple higher than that of <i>C. sinensis</i> . But existence of Cordycepin in <i>C.</i>

	<i>ophioglossoides</i> is not yet confirmed.
Active compound: Adenosine:	Higher in <i>C. militaris</i> and <i>C. ophioglossoides</i> than <i>C. sinensis</i> .
Active compound: Ophiocordin (balanol)	A potent protein kinase inhibitor, is contained in <i>C. ophioglossoides</i> only.

Example 11: Preparation of Paste Form of *Ganoderma*

- 5 The same method as the above example was employed, with a difference that *Ganoderma* was used instead of *Cordyceps*.

The product specification of the *Ganoderma* paste is shown in Table 15.

10

Table 15: Product specification of *Ganoderma* in paste form

Product description	<i>Ganoderma</i> produced by submerged cultivation, followed by filtration, packaging and retorting at 110 -121°C.
Packaging form	Paste form of products, quantity in 3 kg or customised size, packed in laminated retort pouch.
Product fingerprints Proteins/glycoproteins: Overall:	▪ SDS-PAGE and western blotting ▪ Fourier transform infrared (FTIR) spectroscopy
Physical appearance: Colour: Odour: Property:	Brown or light brown Slightly fermented Paste
pH (in 10% solution):	4.5-5.5
Total solid %:	10-15 %
Components (dry basis): Total protein: Total water soluble polysaccharides:	28-30% 15% or above
Ash:	Less than 7%

Heavy metal Arsenic Copper Lead Mercury	In compliance with the Singapore Medicine Act < 0.01 ppm < 15 ppm < 0.2 ppm < 0.01 ppm
Microbial limit: Total bacterial count, CFU/g Total yeast & mould, CFU/g Total E. coli, per gram Staphylococcus aureus, per gram Salmonella spp., per gram	In compliance with the Singapore Medicine Act < 10 ² < 10 ² Absent Absent Absent

Example 12: Manufacturing of Cordyceps “fortified” milk

- 5 The manufacturing trial of Cordyceps fortified UHT milk was conducted in a standard Tetra Pak UHT (ultra-high temperature) plant.

300 liters of fresh milk was loaded into a mixing tank – Tetra Almix, in which 1875 grams of Cordyceps paste with a solid content of 12% was added to make
10 a content of 750 mg Cordyceps per liter of milk. After mixing thoroughly, the milk was pre-heated to 75°C before pumped to the high pressure homogeniser, where it was homogenized at a pressure of 200-250 bar. The preheated, homogenized milk was pumped to the heating section of the UHT unit where it was heated to 140°C for 4 seconds then cooled to 25°C. The cooled milk was
15 pumped to an aseptic buffer tank from which an aseptic packaging line continued to produce finished Cordyceps milk in 250 ml packs.

During the mixing process in the Tetra Almix, the Cordyceps paste was well dispersed into the fresh milk. No fouling was detected in the UHT process (The
20 “fouling” is referred to a problem in dairy flow in pipes or flow in ultra-filtration, where organic particular matters are blocked in pipe and cause the processing and hygienic trouble). As shown in the Table 18, there was no significant

difference of the pH reading and the brix content for the “Before UHT” and “After UHT” (“brix”, related to % total solid, is based on refractive index). The sedimentation content was less than 0.5% v/v. The increase in viscosity was also insignificant (i.e. from 2 to 3 cps).

5

In aspects of sensory, the product had a fresh taste, smooth and creamy mouthfeel.

The incubation test for the products at 35°C and 55°C for a period of seven days showed normal and satisfactory results. The shelf life test for the products at ambient temperature for a period of nine months showed no growth of microbial counts and an acceptable sensory result against ordinary UHT milk. The analysis of the obtained product is shown in Table 16.

15 **Table 16: Product Analysis Record Sheet**

Raw Milk	pH	°Brix	Sedimentation % v/v
	6.53	13.1	< 0.5
UHT Process Cordyceps Milk	Test	Before UHT	After UHT
	pH	6.59	6.50
	°Brix	14.0	14.9
	Sedimentation % v/v	< 0.5	< 0.5
	Viscosity cps	2	3
Finished Product Evaluation	Test		Sensory
	pH	6.48	Fresh milk taste, smooth and creamy mouthfeel
	°Brix	15.0	
	Sedimentation % v/v	0.5	

Example 13: Outline of the general method for the preparation of “fortified” milk

20 An outline of a general method for the preparation of fortified milk according to the invention is shown in Figure 11. The steps are as follows.

I) Step (1)

Fresh milk refers to any kind of liquid milk, such as whole milk, skim milk, reconstituted milk, and the like.

5 II) Step (2)

Pasted ingredients refers to any kind of active pasted ingredients made according to the present invention

10 III) Step (3) & (4)

The mixing tank is any kind of the stirred device used to well mix liquid milk with paste of the invention. This process makes sure that the paste is well dispersed into liquid milk.

15

IV) Step (5)

Mixing the milk + paste mixture and heating to 75°C prior to UHT treatment.

20 V) Step (6)

In addition to breaking down the fat globules in the milk in order to reduce creaming. Homogenization, as a standard industrial process, is also used to disintegrate or finely distribute the particles or fragments of the paste.

25

VI) Step (7), (8) & (9)

UHT treatment refers to a process in any kind of UHT plant where the mixture is pumped through a closed system and treated at 135-150°C for a few seconds, by either indirect heating, direct steam injection or infusion, before being cooled down to 75°C and subsequently to 25°C.

30

VII) Step (10)

Any kind of aseptic packaging machine together with aseptic holding tank is used to pack the obtained fortified milk into different types of UHT milk products with a long shelf life.

VIII) Step (11)

Different from UHT treatment, a standard pasteurization process can be used to prepare the pasteurized fortified milk with a short shelf life.

Example 14: Preparation of "fortified" milk by Cordyceps/Ganoderma extracts

Extract of the Cordyceps and/or Ganoderma mentioned in Example 6 were used to fortify dairy products and drinks. Milk was added to extracts of Cordyceps or Ganoderma in the same way as indicated in the method described in Examples 12 and 13.

Example 15: Preparation of "fortified" milk by using powder made from Cordyceps/Ganoderma spores

Spores of Ganoderma or Cordyceps obtained according to Examples 7 or 8, respectively, were collected, filtered and dried, for example by using a double-cone rotary vacuum dryer or by freeze-drying. The obtained powder was then mixed to milk according to Examples 12 and 13 for the preparation of a milk composition according to the invention.

Example 16: Preparation of "fortified" milk by using paste made from Cordyceps/Ganoderma spores

Spores of *Ganoderma* or *Cordyceps* obtained according to Examples 7 or 8, respectively, were collected, filtered and worked according to Examples 10 or 11. The paste preparation was then mixed to form milk according to Examples 12 and 13.

5

Details of laboratory tests: Profiling of proteoglycan or glycoprotein in *Ganoderma lucidum* and *Cordyceps sinensis* by SDS-PAGE and Western Blotting

- 10 To date, many studies have indicated that one group of physiologically or medicinally active compounds from *Ganoderma* and *Cordyceps* are protein bound glycans and/or polysaccharides [Y.Xu, Intl. J. Food Sci. Tech., 36, 229-242 (2001)]. Glycoproteins with a molecular weight (MW) of 12-18 kilo Dalton (kDa) in *Ganoderma lucidum* were found to have immunosuppressive activities
- 15 [H. Tsunoo, *et al.* US Patent 5,334,704 (1994)] or anti-tumour effects [J.Zhang *et al.* Bio. Biotech. Biochem., 58, 1202-1205 (1994)]. The polysaccharide fraction of *Cordyceps sinensis* was found to have anti-tumour activity capable of suppressing the growth of mouse Sarcoma 180 in vivo [YJ. Chen, Life Sciences, 60, 2349-2359 (1997)].

20

- SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins in the extracts obtained by the methods as described, based primarily on their molecular weights. SDS- polyacrylamide gels with 12% of acrylamide were prepared according to standard procedures (as described by Bio-Rad).
- 25 Electrophoresis was carried out by Mini-Protean® 3 Cell, Bio-Rad in a buffer system (lower and upper): 0.025 M Tris, 0.192M Glycine and 0.1% SDS, at pH 8.3. The extracts (0.5 ml) were mixed with 0.5 ml of treatment buffer containing 0.125 M Tris-Cl, 4% SDS and 20% v/v Glycerol, 0.2 M DTT and 0.02% Bromophenol Blue, at pH 6.8. The mixture (20 µl) was loaded onto the gel and
- 30 electrophoresed at a constant 300 volts, 15mA, for about 2 hours. The gels were then agitated for at least 2 hrs in a fixing solution composed of 50% methanol, 10% acetic acid and 40% water (v/v), and then stained for 4 hrs with

slow agitation in a staining solution containing 50% methanol, 0.05% Coomassie, 10% acetic acid and 40% water (v/v). Gels were de-stained in a solution of 7% acetic acid, 5% methanol, and 88% water (v/v), and preserved in the solution of 7% acetic acid, 5% methanol, and 2% glycerol (v/v). Gels were
5 covered by cellulose films and air-dried.

Western Blotting was performed by transferring the bands on the SDS- PAGE gel onto a nitrocellulose membrane using Mini Trans-Blot® Electrophoretic Transfer Cell, Bio-Rad in a buffer containing 25 mM Tris, 192 mM Glycine, pH
10 8.3, at central voltage 30 V and current limit of 90 mA for at least 18 hours. Transferred membrane was stained by Bio-Rad's Immun-Blot® Kit to detect glycoproteins.

Fig. 6 and 7 show profiling of proteins, glycoproteins and polysaccharides found
15 in the active ingredients of *Ganoderma lucidum* and *Cordyceps sinensis*.

Acute oral toxicity test on mice

1) 10 male and 10 female Sprague Dawley (SD) rats, 6-8 weeks old, were
20 obtained from the Laboratory Animals Centre, National University of Singapore (NUS) and acclimatised at Animal Holding Unit, Kent Ridge, Singapore, for 5 days. Two or three animals were caged together according to their sex. The room was kept at 22-26°C, the humidity at 40-70% and the room was subjected to a 12 hour light and 12 hour dark cycle. The animals were fed
25 conventional laboratory diet and with unlimited supply of drinking water. Before the test substance was orally administered, the feed was withheld overnight. Body weights were recorded just before dosing.

2) 5 male and 5 female rats were then randomly chosen and allocated to be the
30 treatment group. They were dosed by gavage with "Powders of Cordyceps and Ganoderma, 2g/20ml suspension" to give a dose equivalent of 2g/kg rat.

3) The other 10 rats were used as the control group and were dosed with water, at 20 ml/kg rat.

4) All animals were observed 2 hours after dosing, and then hourly for the next 2 hours. Thereafter, observations were made at least once daily up to 15 days. Body weights, amount of feed and level of water consumption were monitored.

5) After 15 days, the body weights were recorded and the rats were euthanised. Post mortem was performed and gross pathology observations were made.

10

The LD₅₀ for powders of *Ganoderma* and *Cordyceps*, determined by the "Fixed Dose Method", was considered to be greater than 2g/kg body weight. No toxicity was observed when the invention substances were administered orally to mice. The procedure adopted was a modification to OECD Acute Oral Toxicity - Fixed Dose Method TG 420. A dose of 2g/kg body weight was used for oral administration.

15

Immuno-modulating test of *Cordyceps* substances on mice

20 1) Methodology

i) Preparation of ethanol extract of *Cordyceps sinensis*

Ethanol extract of *Cordyceps* mycelia, similar to what was described in Example 6, was used to study the immuno-modulation of mouse.

25

Method:

200 grams of powdered *Cordyceps* mycelia were immersed in 800 ml of 80% ethanol solution. The mixture was shaken overnight, and repeated several times until the mycelia were colourless.

30

The residues were removed by centrifugation at 500 rpm for 5 min. Ethanol was removed by a rotary vacuum evaporator at a water bath temperature of 40-50°C.

- 5 The extract was freeze-dried over night to prepare the powdered ethanol extract of *Cordyceps mycelia* for animal studies.

ii) Protocols for evaluating immunomodulating effects in mice

10

Protocol I: In-vivo studies:

To investigate whether the sample stimulated macrophage activity in mice, two doses of each sample were dissolved in water and each was given orally once
15 a day to 5 mice for 14 days. 5 control mice were given only water (control group). At the end of the treatment period, all the mice were killed and macrophages were removed from the peritoneal cavity with PBS.

The macrophages from the control and treated groups were prepared and
20 incubated for 24 hours together with cancer cells at different effectors: target ratios (10:1, 5:1, 2:1 and 1:1).

After the incubation period, the cell mixtures were treated with MTT and measured using a micro-plate reader. The results obtained were analyzed to
25 determine whether significant differences were found.

Protocol II: In vitro studies:

The spleens from untreated mice were removed and a single cell suspension of
30 splenocytes was prepared. The splenocytes were introduced into 96-well microplates and grouped as follows:

- i) Negative control: cells were treated with saline.
 - ii) Positive control: cells were treated with the immunostimulants, LPS and Con-A.
 - iii) Treatment 1: cells were treated with different doses of sample.
 - 5 iv) Treatment 2: cells were treated with different doses of sample and sub-optimal doses of Con A or LPS.
 - v) Treatment 3: cells were treated with different doses of sample and optimal doses of Con A or LPS.
- 10 The cells were incubated for 48 hours. After the incubation period, the cells were treated with MTT to evaluate the activation of splenocytes by the sample.

The values obtained from the various groups were analyzed to determine the statistical significance of the differences.

15

Abbreviations:

PBS: Phosphate Buffered Saline

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Con A: Concanavalin A

20 LPS: Lipopolysaccharide

2) Results

- i) Protocol I: *In-vivo* immuno-modulating test in mice
- 25 Treatment of mice with 80% ethanolic extract of *Cordyceps mycelia* (at 10 mg/kg bw and 100 mg/kg bw doses) resulted in stimulation of macrophages activity when tested *in vitro* at low macrophage: cancer ratio of 1: 1 and 2: 1. Macrophage activity of mice treated with 10 mg/kg bw of the extract was also enhanced when tested at the highest ratio of 10: 1.

30

Protocol II: *In-vitro* immuno-modulating test in mice:

In the absence of the mitogens, ConA and LPS, 80% ethanolic extract of *Cordyceps* mycelia concentration-dependently and significantly increased lymphocyte proliferation *in vitro*. In the presence of sub- optimal and optimal amounts of Con A, it significantly increased T- lymphocyte activity only at the
5 highest concentration tested (10 µg/ml). At this concentration, the extract significantly increased B- lymphocyte proliferation only in the presence of optimal amounts of the LPS mitogen.

Claims

1. An apparatus for large scale production of fungi, comprising:
a bio-reactor; and
5 a liquid medium including water having at least one carbon source and a
volume of at least 500 litres and a pH of at least pH 5; and
means for agitating the liquid medium; and
means for maintaining the temperature of the liquid medium at a
temperature of at least 17°; and
10 means for aerating the liquid medium at a rate of at least 1:0.5 v/v/m; and
anti foaming agent.
2. The apparatus of Claim 1 wherein the means for agitating is a stirrer;
wherein
15 the stirring speed is at least 100 rpm.
3. The apparatus of Claim 1 wherein the liquid medium further comprises
yeast extract.
- 20 4. The apparatus of Claim 1 wherein the liquid medium further comprises
yeast extract having a concentration of at least 15 g/l.
5. The apparatus of Claim 1 wherein the liquid medium further comprises a
peptone source.
25
6. The apparatus of Claim 1 wherein the liquid medium further comprises a
peptone source having a concentration of at least 15 g/l.
7. The apparatus of Claim 1 wherein the liquid medium further comprises
30 glucose as one of the carbon sources.

8. The apparatus of Claim 1 wherein the liquid medium further comprises glucose having a concentration of at least 30 g/l as one of the carbon sources.
9. The apparatus of Claim 1 wherein the liquid medium further comprises maltose as one of the carbon sources.
10. The apparatus of Claim 1 wherein the liquid medium further comprises maltose having a concentration of at least 30 g/l as one of the carbon sources.
11. The apparatus of Claim 1 wherein the liquid medium further comprises at least one mineral source.
12. The apparatus of Claim 1 wherein the liquid medium further comprises magnesium sulphate as a mineral source.
13. The apparatus of Claim 1 wherein the liquid medium further comprises magnesium sulphate having a concentration of at least 0.05 g/l as a mineral source.
14. The apparatus of Claim 1 wherein the liquid medium further comprises monobasic potassium phosphate as a mineral source.
15. The apparatus of Claim 1 wherein the liquid medium further comprises monobasic potassium phosphate having a concentration of at least 0.05 g/l as a mineral source.
16. The apparatus of any of the preceding claims wherein the fungi produced by the large scale production are Cordyceps.
17. The apparatus of any of the preceding claims wherein the fungi produced by the large scale production are Ganoderma.

18. The apparatus of claims 16 or 17 wherein the temperature of the liquid medium is at least 17°C.
19. The apparatus of any of claims 1 to 11 wherein the fungi are *Cordyceps*
5 *militaris*.
20. The apparatus of any of claims 1 to 11 wherein the fungi are *Ganoderma japonicum*.
- 10 21. The apparatus of claim 19 or 20 wherein the mineral comprises sodium nitrate.
22. The apparatus of claim 21 wherein the concentration of sodium nitrate is at least 0.05 g/l.
- 15 23. *Cordyceps* which is obtained by the apparatus of claim 1.
24. *Ganoderma* which is obtained by the apparatus of claim 1.
- 20 25. A multi-stage method for cultivating fungi in a liquid medium comprising:
providing a source of fungi;
inoculating a first stage liquid medium culture using the fungi;
agitating the first stage liquid medium culture while letting the fungi grow
for a period of time;
- 25 inoculating a second stage liquid medium using the first stage culture;
agitating the second stage liquid medium while letting the culture grow for
a period of time;
- inoculating a third stage liquid medium of at least 500 litres contained in a
bio-reactor using the second stage culture;
- 30 agitating the third stage liquid medium while letting the culture grow for a
period of time;

maintaining the liquid medium of each stage at a pH of at least approximately pH 5;

adding at least one carbon source to the liquid medium of each stage.

- 5 26. The method of Claim 25 further comprising steps for adding glucose, maltose, monobasic potassium phosphate, magnesium sulphate and yeast extract into the second or third stage liquid medium.
- 10 27. The method of Claim 25 further comprising adding yeast extract to the second or third stage liquid medium having a concentration of at least 15 g/l.
28. The method of any of the preceding claims wherein the yeast extract contains vitamins.
- 15 29. The method of Claim 25 further comprising the step of adding at least one type of mineral to the second or third stage liquid medium.
30. The method of Claim 29 wherein the mineral comprises magnesium sulphate.
- 20 31. The method of Claim 29 wherein the mineral comprises monobasic potassium phosphate.
32. The method of Claim 29 further comprising adding magnesium sulphate to the second or third stage liquid medium having a concentration of at least 0.05 g/l.
- 25 33. The method of Claim 29 further comprising the step of adding monobasic potassium phosphate to the second or third stage liquid medium such that it has a concentration of at least 0.05 g/l.
- 30 34. The method of Claim 25 further comprising:

culturing the fungi in first stage liquid medium for a period of at least 48 hours; wherein

the liquid medium has a volume of at least approximately 500 ml.

5 35. The method of Claim 25 wherein the step of agitating the first stage liquid medium comprises the step of rotating the container of the medium at a rate of at least 100 rpm.

10 36. The method of Claim 25 further comprising:
aerating the second stage liquid medium at a rate of at least 1:0.5 v/v/m;
and
culturing the fungi in a volume of at least approximately 50 ml of the second stage medium for at least 40 hours.

15 37. The method of Claim 25 wherein the step of agitating the second stage liquid medium comprises stirring the second stage liquid medium at a speed of at least 100 rpm.

20 38. The method of Claim 25 further comprising the steps of:
aerating the third liquid medium at a rate of at least 1:0.5 v/v/min; and
culturing the fungi in a volume of at least approximately 500 litres of the third stage medium for at least 48 hours.

25 39. The method of any preceding claims wherein the fungi is comprised of Cordyceps.

40. The method of any preceding claims wherein the fungi is comprised of Ganoderma.

30 41. The methods of any of claims 39 to 40 further comprising the step of maintaining a temperature of at least 17°C in the liquid medium.

42. The method of claims 25 to 38 wherein the fungi is comprised of *Cordyceps militaris*; and
further comprising the step of maintaining the temperature of the liquid medium at least 17°C.
- 5
43. A method for the culture of *Cordyceps militaris* comprising growing the *Cordyceps militaris* on a medium comprising sodium nitrate.
44. The method of Claim 43 wherein the concentration of sodium nitrate in
10 the medium is at least 0.5%.
45. A method for the culture of *Cordyceps militaris* comprising growing the *Cordyceps militaris* in a medium comprising Riboflavin (vitamin B2).
- 15 46. The method of Claim 45 wherein the concentration of Riboflavin in the medium is at least 0.01%.
47. Cordyceps which is obtained by the method of claims 25-39 or 41-46.
- 20 48. Ganoderma which is obtained by the method of claims 25-38 and 40-41.
49. A growth medium for culturing Cordyceps or Ganoderma comprising:
glucose; and
maltose; and
25 water.
50. A growth medium as claimed in Claim 49 further comprising yeast extract.
- 30 51. A growth medium as claimed in Claim 50 wherein the yeast extract comprises vitamins.

52. A growth medium as claimed in Claim 50 or 51 wherein the yeast extract has the concentration of at least 15 g/l.
53. A growth medium as claimed in Claim 49 wherein the glucose has the
5 concentration of at least 30 g/l.
54. A growth medium as claimed in Claim 49 wherein the maltose has the concentration of at least 30 g/l.
- 10 55. A growth medium as claimed in Claim 49 further comprises minerals.
56. A growth medium as claimed in Claim 55 wherein the minerals include sodium phosphate.
- 15 57. A growth medium as claimed in Claim 55 wherein the minerals include monobasic sodium phosphate.
58. A growth medium as claimed in Claim 55 wherein the minerals include dibasic sodium phosphate.
20
59. A growth medium as claimed in Claim 56, 57 or 58 wherein the mineral has the concentration of at least 1 g/l.
60. A growth medium as claimed in Claim 55 wherein the minerals include
25 magnesium sulphate.
61. A growth medium as claimed in Claim 60 wherein the magnesium sulphate has the concentration of at least 0.05 g/l.
- 30 62. A growth medium as claimed in Claim 55 wherein the minerals include sodium nitrate.

63. A growth medium as claimed in Claim 62 wherein the sodium nitrate has a concentration of at least 0.5 g/l.
64. A method of producing spores of Ganoderma and/or Cordyceps comprising:
5 applying a pressure of at least 2 bar to a culture of Ganoderma and/or Cordyceps.
65. The method of Claim 64 further comprising culturing Ganoderma and/or
10 Cordyceps in a bio-reactor system.
66. The method of Claim 65 further comprising culturing Ganoderma and/or Cordyceps in a system comprising: a bio-reactor; and
15 a liquid medium including water having at least one carbon source and a volume of at least 500 litres and a pH of at least pH 5; and
means for agitating the liquid medium; and
means for maintaining the temperature of the liquid medium at least 17°;
and
20 means for aerating the liquid medium at a rate of at least 1:0.5 v/v/m; and anti foaming agent.
67. Ganoderma and/or Cordyceps spores which is obtained by the methods of claim 64 to claim 66.
- 25 68. A method of extracting ingredients of Cordyceps comprising:
soaking Cordyceps mycelia in hot water having a temperature of more than 90°C for at least 12 hours to obtain a mixture;
filtering the mixture into a filtrate portion and a residue portion;
mixing the filtrate with a solvent to obtain a precipitate of water soluble
30 extract;
mixing the residue with a solvent and agitating gently;
removing the solvent to obtain a solvent soluble extract.

69. A method according to Claim 68 wherein the solvent is an organic solvent.
- 5 70. A method according to Claim 69 wherein the organic solvent is ethanol.
71. A method according to Claim 68 wherein the water is mixed with an alkaline buffer.
- 10 72. A method according to Claim 71 wherein the water includes of an alkaline buffer of pH10.
73. A method according to Claim 68 further comprising the steps of:
performing three more repetitions of the steps of mixing the residue with
15 a solvent and agitating gently before removing the solvent to obtain the solvent-soluble extract.
74. The method according to any one of claims 25-46, further comprising the
step of collecting, filtering the mycelium, and washing the mycelium
20 removing the residues of the medium present in the culture.
75. Cordyceps and/or Ganoderma paste prepared according to the method of claim 74.
- 25 76. Cordyceps comprising at least the following components: 27-28% of total protein, at least 20% of total water soluble polysaccharides, at least 5% of cordycepic acid, at least 0.1% of cordycepin and at least 0.15% of adenosine.
- 30 77. A Cordyceps paste comprising the Cordyceps characterised in claim 76.

78. Ganoderma comprising at least the following components 28-30% of total protein, and at least 15% of total water soluble polysaccharides.
- 5 79. A method for preparing purified Cordyceps and/or Ganoderma paste comprising culturing Cordyceps and/or Ganoderma in presence of the growth medium of claims 49-63.
- 10 80. The method of claim 79, further comprising the steps of collecting, filtering the mycelium, and washing the mycelium removing the residues of the medium present in the culture.
81. A Cordyceps and/or Ganoderma paste prepared according to the method of any one of claims 79-80.
- 15 82. A unicellular Cordyceps and/or Ganoderma.
83. A unicellular culture comprising substantially unicellular strain Cordyceps and/or Ganoderma.
- 20 84. The unicellular culture of claim 83, comprising substantially 100% of unicellular strain of Ganoderma and/or Cordyceps.
- 25 85. A beverage and/or food comprising at least one of the following: the Cordyceps of claim 23, the Ganoderma of claim 24, the Cordyceps of claim 47, the Ganoderma of claim 48, Ganoderma and/or Cordyceps spores of claim 67, the Ganoderma and/or Cordyceps paste of claim 78, the unicellular Ganoderma and/or Cordyceps of claim 82, and the unicellular culture of claims 83-84.
- 30 86. A fortified milk product comprising mixing milk with at least one of the following: the Cordyceps of claim 23, the Ganoderma of claim 24, the Cordyceps of claim 47, the Ganoderma of claim 48, Ganoderma and/or

Cordyceps spores of claim 67, the Ganoderma and/or Cordyceps paste of claims 77-78, the unicellular Ganoderma and/or Cordyceps of claim 82, and the unicellular culture of claims 83-84.

- 5 87. A method for the preparation of fortified milk product comprising mixing milk to at least one of the following: the Cordyceps of claim 23, the Ganoderma of claim 24, the Cordyceps of claim 47, the Ganoderma of claim 48, Ganoderma and/or Cordyceps spores of claim 67, the Ganoderma and/or Cordyceps paste of claims 77-78, and the unicellular Ganoderma and/or
10 Cordyceps of claim 82, and the unicellular culture of claims 83-84.
88. A pharmaceutical composition comprising at least one of the following: the Cordyceps of claim 23, the Ganoderma of claim 24, the Cordyceps of claim 47, the Ganoderma of claim 48, Ganoderma and/or Cordyceps spores of
15 claim 67, the Ganoderma and/or Cordyceps paste of claims 77-78, and the unicellular Ganoderma and/or Cordyceps of claim 82, and the unicellular culture of claims 83-84.
89. The pharmaceutical composition of claim 88, further in combination with a
20 pharmaceutically acceptable eccipient and/or diluent.
90. The use of at least one of the following: the Cordyceps of claim 23, the Ganoderma of claim 24, the Cordyceps of claim 47, the Ganoderma of claim 48, Ganoderma and/or Cordyceps spores of claim 67, and the
25 Ganoderma and/or Cordyceps paste of claims 77-78, for the manufacture of a pharmaceutical composition for the treatment of at least tumors, the stimulation of macrophagies activity, the stimulation of a system, stimulation of kidney and respiratory functions, and enhance the circulatory function of the body.
30
91. At least one Cordyceps selected from the group consisting of: the Cordyceps of claim 23, the Cordyceps of claim 47, the Cordyceps spore(s)

of claim 67, the Cordyceps of claim 76, the Cordyceps paste of claim 75, claim 77 or claim 81, the unicellular Cordyceps of claims 82-84, for use in at least one of the following: in the treatment of tumors, in the stimulation of macrophages activity, and/or in the stimulation of a immunological reaction.

5

92. At least one Ganoderma selected from the group consisting of: the Ganoderma of claim 24, the Ganoderma of claim 48, the Ganoderma spore(s) of claim 67, the Ganoderma of claim 78, the Ganoderma paste of claim 75 or claim 81, and the unicellular Ganoderma of claims 82-84, for use in at least one of the following: in the treatment of tumors, in the stimulation of macrophages activity, and/or in the stimulation of a immunological reaction.

10

93. The food or beverage of claim 85 for use in at least one of the following: in the treatment of tumors, in the stimulation of macrophages activity, and/or in the stimulation of a immunological reaction.

15

94. The fortified milk of claim 86 for use in at least one of the following: in the treatment of tumors, in the stimulation of macrophages activity, and/or in the stimulation of a immunological reaction.

20

95. The pharmaceutical composition of claims 88-89 for use in at least one of the following: in the treatment of tumors, in the stimulation of macrophages activity, and/or in the stimulation of a immunological reaction.

25