

COMMONWEALTH of AUSTRALIA

PATENTS ACT 1952

APPLICATION FOR A STANDARD PATENT

¹
We of Allelix Inc.
6850 Goreway Drive, Mississauga
Ontario L4V 1P1
Canada

613079

hereby apply for the grant of a Standard Patent for an invention entitled:

Maintenance of the viability of microorganisms for use
in microbial inoculants

which is described in the accompanying provisional complete specification.

Details of basic application(s):—

Number

Convention Country

Date

-

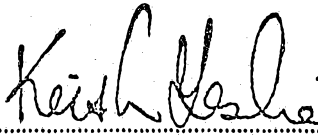
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The address for service is care of DAVIES & COLLISON, Patent Attorneys, of 1 Little
Collins Street, Melbourne, in the State of Victoria, Commonwealth of Australia.

Dated this THIRTIETH day of AUGUST 19 88

To: THE COMMISSIONER OF PATENTS


.....
(a member of the firm of DAVIES &
COLLISON for and on behalf of the Applicant).

Davies & Collison, Melbourne and Canberra.

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

DECLARATION IN SUPPORT OF CONVENTION OR NON-CONVENTION APPLICATION FOR A PATENT

Insert title of invention.
entitled: MAINTENANCE OF THE VIABILITY OF MICROORGANISMS
FOR USE IN MICROBIAL INOCULANTS

Insert full name(s) and address(es)
of declarant(s) being the appli-
cant(s) or person(s) authorized to
sign on behalf of an applicant
company.
~~I~~
~~We~~ Graham Strachan of 118 West Deane Park Drive,
Islington, Ontario M9B 2S3 Canada

Cross out whichever of paragraphs
1(a) or 1(b) does not apply

1(a) relates to application made
by individual(s)
1(b) relates to application made
by company; insert name of
applicant company.

do solemnly and sincerely declare as follows :-

1. (a) ~~I am~~ ~~the applicant~~ ~~XXXXXX~~
~~We are~~

or (b) I am authorized by ALLELIX INC. of 6850 Goreway Drive,
Mississauga, Ontario L4V 1P1, CANADA

Cross out whichever of paragraphs
2(a) or 2(b) does not apply

2(a) relates to application made
by inventor(s)
2(b) relates to application made
by company(s) or person(s) who
are not inventor(s); insert full
name(s) and address(es) of inven-
tors.

the applicant..... for the patent to make this declaration on ^{its}
~~their~~ behalf.

2. (a) ~~I am~~ ~~the actual inventor~~ ~~XXXXXX~~
~~We are~~

or (b)
CHARLEY, Robert C. of 2174 Madden Boulevard,
Oakville L6H 3M2, CANADA

is the actual inventor..... of the invention and the facts upon which the applicant.....
are
is
are entitled to make the application are as follows :-

State manner in which applicant(s)
derive title from inventor(s)

By virtue of an Assignment dated 30 July 1986,
the said actual inventor assigned the invention to the
applicant.

Cross out paragraphs 3 and 4
for non-convention applications.
For convention applications,
insert basic country(s) followed
by date(s) and basic applicant(s).

3. ~~The basic application~~ ~~XXXXXX~~ ~~as defined by Section 141 of the Act~~ ~~was~~
~~XXXXXX~~ ~~made~~ ~~XXXXXX~~
in on the
by
in on the
by
in on the
by

4 ~~XXXXXX~~ ~~The basic application~~ ~~XXXXXX~~ ~~referred to in paragraph 3 of this Declaration~~ ~~was~~
~~XXXXXX~~ ~~made in a Convention country in respect of the invention the subject~~ ~~XXXXXX~~
~~of the application~~

Insert place and date of signature.

Declared at ~~Mississauga~~ this ~~26~~ day of ~~August~~, 1988

Signature of declarant(s) (no
attestation required)

ALLELIX INC.

Note: Initial all alterations.

By: ~~Alles~~

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MAINTENANCE OF THE VIABILITY OF MICROORGANISMS FOR USE IN MICROBIAL INOCULANTS

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(56) Prior Art Documents
US 4155737
WO 85/02972
FR 2469861

(57) Claim

1. A method for maintaining the viability of bacterial cells having a beneficial effect on plants comprising maintaining a suspension of the bacterial cells in a free-flowing composition comprising a water dispersible, substantially soluble, non-crosslinked polysaccharide.

2. A method for inoculating a plant seed with bacterial cells having a beneficial effect on plants comprising contacting the plant seed with a free flowing composition which comprises (i) a suspension of the bacterial cells and (ii) a water dispersible, substantially soluble, non-crosslinked polysaccharide.

9. A composition capable of maintaining the viability of bacterial cells having a beneficial effect on plants comprising bacterial cells and a water dispersible substantially soluble non-crosslinked polysaccharide, the composition being a optionally dried free flowing suspension.

10. A plant seed composition capable of inoculating plant seed with bacterial cells having a beneficial effect on plants, the composition comprising plant seed, the bacterial cells and an optionally dried free flowing suspension of a water dispersible, substantially soluble non-crosslinked polysaccharide.

613079

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COMPLETE SPECIFICATION

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COMPLETE SPECIFICATION FOR THE INVENTION ENTITLED:

Maintenance of the viability of microorganisms for use in microbial inoculants

The following statement is a full description of this invention, including the best method of performing it known to me/us:-

-1A-

The invention pertains to a method for stabilizing bacterial cells for extended periods of time. This stabilization is achieved through the formation of a slurry containing the bacterial cells, and non-cross-linked agents such as polysaccharides. The invention further pertains to a composition of matter containing non-crosslinked sodium alginate and microorganisms in suspension. The invention also pertains to the above composition which additionally contains oils in emulsions. Additionally, the invention pertains to the composition and use a dry powder which could be used to coat seeds. The invention further pertains to the use of such dry powders as microbial inoculants in agriculture. The invention also pertains to the use of sodium alginate and microorganisms in solution, dried to a powder and used to coat seeds directly or when suspended in oil.

The use of microbial inoculants (such as Rhizobium) has long been known to increase crop yields. The utility of this approach has been limited by the availability of compositions capable of both affixing the microbial cells to the seed and maintaining the viability of the microbial inoculant. Burton, J.C. (U.S. Patent 2,995,867) discloses the possibility of using water soluble gums such as methylcellulose as a seed coating. Through the use of such coatings, it is possible to keep microbial cells alive and effective for over two weeks.

The success of this work spurred others to attempt to identify alternate compositions which might be capable of maintaining bacterial viability for longer periods of time. A method was disclosed by Hamrin, B.S.A. (U.S. Patent 3,688,437), which involved the use of seed coatings comprising a gelatin-calcium alginate foam. Dommergues, Y.R., et al. (U.S. Patent 4,155,737) disclosed the possibility of embedding the microbial inoculant in a polyacrylamide seed coating.

The possibility of stabilizing bacterial cells in a microbial inoculant through the use of a polysaccharide matrix was shown by Mugnier, J., et al. (French Patent 2,469,861). This patent discloses the ability to stabilize a suspension of bacterial cells by embedding them in an alginate gel. The gel disclosed in this patent is formed by the addition of calcium chloride to a solution containing a suspension of bacterial cells and sodium alginate. The addition of calcium chloride results in the precipitation and cross-linking of the previously soluble sodium alginate mixture.

A similar alginate composition is disclosed by Jung, G. (European Patent 17,565 and U.S. Patent 4,434,231). These patents disclose the possibility of crosslinking or precipitating solutions of sodium alginate that contain suspensions of bacterial cells. The Jung patents disclose the use of calcium sulfate to convert a sodium alginate suspension into a gel. This crosslinking reaction can be delayed through the addition of sodium phosphate to the suspension. Bacterial cells treated in this manner remained viable for greater than 60 days. These patents further disclose the use of such embedded microorganisms as microbial inoculants in agriculture. Although the ease of formation of crosslinked alginate gels commended their use as an embedding matrix for a microbial inoculant, such matrices were found to be inferior to embedding matrices of xanthan gums in that embedding matrices composed of xanthan gums were found to release microorganisms into the soil more readily than matrices composed of alginates. (Jung, G., et al., Plant and Soil, 65:219-231 (1982)).

Jung, et al. (European patent no. 83267) disclose compositions containing microbial cells and polymeric gels which may be dried and used to inoculate plant seeds. The patent further discloses the use of a crosslinked alginate suspension of microbial cells as a microbial inoculant.

One concern reflected in the prior art is that suitable embedding agents should provide an environment strong enough to resist external abrasion and adverse forces yet be pliable enough to allow release of internal components and permit breakdown of the

matrix at the appropriate time. Two different methods have been disclosed in order to accomplish this goal. Redenbaugh, M.K., et al. (P.C.T. Publication WO85/02972) disclosed the use of crosslinked alginate-bacterial cell suspensions which are surrounded by an external membrane as a microbial inoculant. Fravel, D.R., et al. (Phytopathology, 75:774-777 (1985)) disclosed the use of crosslinked alginate-bacterial cell suspensions which contain clays or other materials, added as bulking agents. The process disclosed by Fravel, et al produces solid pellets of relatively uniform size.

Thus, the prior art teaches the value of pre-inoculating seeds with microbial suspensions in order to enhance their germination and growth. A wide variety of different embedding matrices have been disclosed in the prior art. Especially significant are matrix compositions which employ crosslinked or precipitated alginates or very thick, gum-like compositions. Such compositions have been successful in sustaining bacterial cell viability for extended periods of time, while simultaneously possessing the desired properties of rigidity and support. The preparation of gels, however, requires additional steps and both gels and gums involve handling difficulties.

The present invention relates to an improved process for maintaining cell viability and cellular integrity under conditions (temperature, pH, enzymatic reactions, etc) under which cell viability and integrity would normally deteriorate. The invention is useful in the formation of microbial inoculants in agriculture.

The invention utilizes the properties of a liquid system wherein the cells are protected from environmental conditions, but freely accessible to substrates and co-factors, which may be added at any time to the system. The liquid system described by the invention may have varying degrees of fluidity, but does not have semi-solid, viscous, gel- or gum-like properties.

The invention is based on the discovery that non-crosslinked polysaccharide solutions stabilize the viability of non-dormant microbial cells. Such solutions can also, in accord with the present invention, be used to preserve dormant microbial cells for subsequent use.

In detail, the invention pertains to a method for maintaining the viability of bacterial cells having a beneficial effect on plants which comprises: maintaining a suspension of the bacterial cells in a free-flowing composition which comprises a water dispersible, substantially soluble, non-crosslinked polysaccharide.

Additionally, the invention pertains to a method for inoculating a plant seed with a microorganism which comprises: providing to the plant seed a free flowing composition which comprises (i) a suspension of the microorganism and (ii) a water dispersible, substantially soluble, non-crosslinked polysaccharide.

The invention is also directed to a method for maintaining the viability of a microorganism which comprises: drying a free-flowing composition, the composition comprising a suspension of the microorganism and a water dispersible, substantially soluble, non-crosslinked polysaccharide to form a dried composition.



The invention is further directed to a method for inoculating a plant seed with a microorganism which comprises:

(a) drying a free-flowing composition, the composition comprising a suspension of the microorganism and a water dispersible, substantially soluble, non-crosslinked polysaccharide, to form a dried composition, and

(b) providing the dried composition (a) to the plant seed.

The invention also relates to a composition capable of maintaining the viability of bacterial cells having a beneficial effect on plants comprising bacterial cells and a water dispersible substantially soluble non-crosslinked polysaccharide, the composition being an optionally dried free flowing suspension.

Accordingly the present invention also encompasses a plant seed composition capable of inoculating plant seed with a microorganism, the composition comprising plant seed, a microorganism and an optionally dried free flowing suspension of a water dispersible, substantially soluble non-crosslinked polysaccharide.

The composition aspects of the present invention may be employed in the method aspects and thus features of the method aspects are applicable to the composition aspects mutatis mutandis.

The present invention relates to suspensions containing polysaccharides, and the use of such suspensions as a means of stabilizing or preserving microbial cells.

The invention further relates to drying a solution which comprises a non-crosslinked polysaccharide, and a microorganism to produce a powder and using the powder obtained after drying either directly or when dispersed in oil.

As used herein, the terms "microorganism", "microbial cells" and "bacterial cells" refer to any of a wide variety of microorganisms. Examples of such microorganisms are Pseudomonas, Serratia, Arthrobacter, Azospirillum, Rhizobium and Bacillus, but the invention can be used to maintain the viability of any microorganism which has a beneficial effect on the growth, emergency, yield, etc. of plants.

The term "polysaccharide", as used in this invention is meant to refer both to polysaccharides which must be externally added to the suspension or micro-



bial cells, and to polysaccharides produced directly by a microorganism under appropriate culture conditions, such as for example by permitting cultures of microorganisms to enter growth limiting conditions (e.g. nitrogen exhaustion) with an excess of carbon, or energy source. (Sutherland, I.W., Microbial Polysaccharides and Polysaccharases, (Berkeley, Gooday, and Ellwood, eds.) Acad. Press NY (1979)). An example of a polysaccharide included in the invention is the polysaccharide obtained from algae. Such polysaccharides are collectively referred to as "alginates." The invention is preferably directed to the use of polysaccharides which are substantially soluble in an aqueous solution. Examples of such polysaccharides are alginates, celluloses, or starches. The soluble polysaccharides of the present invention are further restricted to those which are non-crosslinked.

As used in this invention, a polysaccharide is considered to be "non-crosslinked" if the polysaccharide is either free of intermolecular crosslinks, or if the degree of crosslinking is of such minor character so as to permit the polysaccharide to remain soluble in an aqueous solution. The concentration of polysaccharide in these solutions is described as its concentration relative to that of the total volume of the solution (i.e. as a weight/volume percentage). Unless otherwise indicated the alginate-microorganism mixtures of the present invention are prepared by dissolving alginates in water and then adding the alginate solution to a culture broth. The degree of crosslinking is less than 10%, preferably less than 5%.

The present invention relates to the use of "water dispersible," "free flowing" compositions. These terms are meant to refer to compositions which have low viscosity, and are pourable. These terms are specifically intended to exclude compositions which are solids, gels or gums at ambient (4°C - 30°C) temperatures.

As used herein, the terms "dried" or "drying" is intended to describe the removal of water. Any means capable of achieving the removal of water may be employed. Suitable techniques including air drying, spray drying, lyophilizing, etc. are well known in the art.

As used herein, the term "oil" refers to any of a large class of compounds which are soluble in hydrophobic solvents such as ether or alcohol, but are substantially insoluble in water. The oils suitable for use in the present invention are liquids at ambient temperatures. Examples of suitable oils are: Mineral oil, or vegetable oils such as soya oil, canola oil, corn oil, etc.

As mentioned above, an oil is substantially insoluble in water. Thus a water/oil mixture will tend, upon storage, to spontaneously separate (on the basis of density) into two substantially pure solutions, one of which contains the oil and the second of which contains the water. Although the present invention can be practiced with a water/oil mixture, it is preferable to add an "emulsifier" or "emulsifying agent" to the mixture. An emulsifier is a substance which is capable of enhancing the dispersion of the aqueous phase into the oil. Emulsifiers are disclosed by

Huettinger, R., (Seif., Oele, Fette, Wachse, 109:455-479 (1983)). Examples of suitable emulsifying agents are Arlacel 83TM, Arlacel 186TM, and Tween 81. ArlacelTM is manufactured by ICI Chemical Corp.

The present invention provides a means for stabilizing the viability of microbial cells and may be used to preserve microorganisms for a wide variety of applications. In particular the present invention may be used to maintain viability of microorganisms in inoculants for agricultural and industrial applications. According to the invention cells may be stabilized to provide a suitable storage period and then applied easily to seeds in the form of a liquid, free-flowing coating to produce a variety of effects including enhancement of emergence, growth and yield. The applied microorganisms may function in diverse manners to produce the desired response and include members of the genera Rhizobium, Pseudomonas, Bacillus, Arthrobacter, Serratia and Azospirillum.

The invention can be performed using a large number of possible non-crosslinked polysaccharides, such as starch, or xanthan gum, however it is preferable to use alginate polysaccharides. Although the invention can be practiced using polysaccharide solutions which range from 0.01-20%, it is preferable to use polysaccharides solutions of between 0.5-2.5%, with about 1.0-2.0% being most preferable for alginate.

To produce the polysaccharide-bacterial cell suspensions of the invention, aliquots of a fresh, healthy culture of cells containing a cell concentration high enough to provide any desired biological or in-

dustrial effect, but not so high as to negatively interfere with such effect, and preferably between 1-50 grams of cells (dry weight) per liter, are mixed with equal aliquots of sterile solutions containing a soluble polysaccharide. Bacterial cultures treated in this manner may then be stored at any temperature wherein the bacteria remain viable, such as, e.g., between 4-30° C. The degree of bacterial stabilization has been found to be independent of the sample storage temperature.

The polysaccharide solution may contain nutrients or growth factors such as, for example, sugars, amino acids, proteins, salts, etc. In addition, the polysaccharide solutions may contain osmoregulatory agents, such as buffers, which may be required in order to practice the invention.

In one embodiment of the invention, the bacterial cells are suspended in a polysaccharide solution which may then be applied to seeds, by any of a variety of means, such as, for example, dipping, spray drying, etc. Such techniques are disclosed by Cull, S.W., et al. (European Patent 97,459); Mugnier, J., et al. (French Patent 2,469,861); Jung, G. (U.S. Patent 4,434,231); and Redenbaugh M.K., et al. (PCT No. W085/02972).

In a preferred embodiment of the invention, the bacterial cells are suspended in a non-crosslinked polysaccharide solution and then incorporated into an oil emulsion. This composition may then be used as an agricultural inoculant, for example, by diluting it with water and using it as a liquid spray or by coating it directly onto seeds. The polysaccharide/cell

solution may alternatively be dried to a powder to provide successful storage either alone or, preferably, in combination with agrichemicals. This powder may be applied directly to seeds, or may be dissolved in water or dispersed in oil to produce a liquid which can be sprayed onto seeds.

The previously cited techniques applying polysaccharide/bacterial cell suspensions to seeds can be readily adapted by one of ordinary skill in the art so as to enable their application to the compositions of the present invention. It is, however, preferable to dry the suspensions at 30°C by blowing air over their surfaces. This is preferably accomplished by placing the suspension in an incubator and blowing sterile, filtered air across the surface of the suspension. The dried composition may then be coated onto seeds by mixing.

Having now generally described this invention, the same will be better understood by reference to certain specific examples which are included herein for purposes of illustration only and are not intended to be limiting of the invention, unless specified.

Example 1: Growth of Microbial Strains

Rhizobium japonicum cultures were maintained on YEM agar plates (10g mannitol; 0.5g (Difco) yeast extract; 0.5g K_2HPO_4 ; 0.2g $MgSO_4 \cdot 7H_2O$; 0.1g NaCl; 20g agar; water to 1L. 100 ml liquid cultures were obtained by inoculating 100ml of medium A (10g sucrose; 0.5g (Veeprex B800) yeast autolysate; 0.22g K_2HPO_4 ; 0.1g $MgSO_4 \cdot 7H_2O$; 0.04g $CaCl_2$; 0.02g $FeCl_3$; water to 1L) with a single colony from a YEM agar plate. The li-

quid cultures were incubated at 30°C for 7 days on a orbital shaker at 140 RPM. One liter cultures were obtained by transferring a fresh 100ml culture into 1L of medium A and incubating for 5-7 days at 30°C on an orbital shaker at 140 RPM.

Unless otherwise indicated, all other cultures were maintained on PAF agar plates (38g (Gibco) Pseudomonas agar F; 10g glycerol; 20g agar; water to 1L) liquid cultures were obtained by inoculation of TSB (30 g (Gibco) tryptic soy broth; water to 1L) with a single colony from a PAF agar plate and incubating at 30°C for 48 hours on an orbital shaker at 140 RPM.

Example 2: Effect of Temperature on Stability of Rhizobium japonicum

Rhizobium japonicum USDA 138 was grown as described in Example 1. 50ml of fresh culture medium was mixed with an equal amount of sterile solutions of 1% and 2% non-crosslinked sodium alginate to produce samples having .5% or 1% alginate. Samples containing a final alginate concentration of 1% were stored at 4°C, room temperature, and 30°C. The sample containing 0.5% alginate was stored at room temperature. Periodically, the mixtures were well agitated and samples removed for viable cell culture counts. The result of this experiment is shown in Table 1. This experiment demonstrated that the stability of R. japonicum cells in an alginate slurry was essentially unaffected by storage temperature.

TABLE 1

Log viable count/mL

<u>Sample</u>	<u>to</u>	<u>1wk</u>	<u>4wks</u>	<u>10wks</u>	<u>21wks</u>	<u>26wks</u>
1%; 4°C	8.84	8.45	7.99	9.06	9.59	---
1%; RT	9.61	8.93	9.86	9.44	8.64	---
1%; 30°C	9.42	8.70	8.85	9.06	8.53	8.17
0.5%; RT	10.42	9.68	9.62	9.29	8.86	---

Example 3: Effect of Polysaccharide on Stability of Rhizobium japonicum

Rhizobium japonicum USDA 138 was grown as described in Example 1. Samples of the liquid cultures were removed and mixed with the non-crosslinked polysaccharides shown in Table 2. Mixtures were stored at room temperature and sampled periodically for viable cell counts following vigorous agitation. This experiment showed that a wide variety of soluble polysaccharides could be used to sustain the viability of bacterial cells.

TABLE 2

Sample	to	Log viable count/ML			
		1wk	4wks	10wks	30wks
2.5% Dextran	7.76	10.48	9.14	8.78	8.00
0.25% WaterLok BL00	7.92	9.78	8.37	8.52	6.60
3.5 Corn starch	7.83	9.56	9.93	10.54	9.29
0.5% Carboxymethyl-cellulose	7.88	9.33	9.26	10.84	8.18
0.5% Gum ghatti	7.63	8.51	8.65	8.36	8.31
0.5% Xanthan gum	8.19	9.72	9.39	9.13	8.79
1% Gum arabic	7.77	9.26	9.47	10.21	7.89
0.5% Gelatin	7.73	9.42	9.59	9.04	—
0.5% Alginate/0.5M sorbitol	7.88	9.55	9.03	10.99	9.23
0.5% Alginate/0.5M sucrose	7.36	7.97	9.94	9.00	9.15
0.5% Alginate/0.5% saline	7.62	8.43	7.44	—	0
0.5% Alginate/0.5% yeast autolysate	7.81	8.49	7.29	5.31	0
0.5% Alginate/0.5% activated charcoal	7.79	9.68	8.87	8.14	8.03
0.5% Alginate/14% beet molasses	8.01	8.51	6.61	5.82	0
1.5% Carboxymethyl-cellulose	7.61	9.44	9.49	9.26	7.56
2.5% Alginate	7.71	8.74	9.08	—	—
0.5% Alginate	7.74	9.38	9.00	8.31	7.85
0.05% Alginate	7.77	8.87	9.34	8.33	—
0.5% Alginate + 0.15% B-cyclodextrin	7.71	8.97	9.24	8.61	7.94

Example 4: Stability of Pseudomonas and Bacillus
Cultures in Alginate Slurries

50ml of fresh healthy cultures of Pseudomonas fluorescens 17-34, Pseudomonas putida 1-104, Pseudomonas sp 55-14, Pseudomonas sp. 67-4, Bacillus sp. 86-64 and Pseudomonas putida G2-8 were grown as described in Example 1 and mixed with equal aliquots of sterile 1% non-crosslinked alginate solutions. The mixtures were stored at room temperature and periodically agitated and sampled for viable cell counts. The results of this experiment are shown in Table 3. This experiment shows that 1% alginate slurries are successful in stabilizing a variety of bacterial strains.

TABLE 3

<u>Organism</u>	<u>T₀</u>	<u>Log viable count/ML</u>				
		<u>1wk</u>	<u>4wks</u>	<u>8wks</u>	<u>10wks</u>	<u>34wks</u>
<u>P. fluorescens</u> 17-34	11.88	11.83	11.02	8.16	7.43	7.00
<u>P. putida</u> 1-104	10.19	11.33	10.32	8.27	7.45	7.40
<u>Pseudomonas</u> sp. 55-14	11.80	11.08	9.62	8.76	6.61	7.22
<u>Pseudomonas</u> sp. 67-4	11.85	12.04	10.27	8.22	7.21	8.29
<u>Bacillus</u> sp. 86-64	9.49	9.86	9.36	7.79	7.03	8.20
<u>P. putida</u> G2-8	10.41	11.73	11.48	8.26	7.25	8.26

Example 5: Effect of Storage in Sealed Vials on the Stability of Pseudomonas and Bacillus Strains in Alginate Solutions

Aliquots of fresh healthy cultures of Pseudomonas fluorescens 17-34, Pseudomonas putida 1-104, Pseudomonas sp. 55-14, Pseudomonas sp. 67-4, Bacillus sp. 86-64 and Pseudomonas putida G-28 were grown as described in Example 1. Aliquots were mixed with equal volumes of a sterile 1% non-crosslinked alginate solution and dispensed into sterile 2.2ml glass vials. The vials were completely filled and tightly capped, and were then stored at 30°C or room temperature. Periodically samples were agitated and viable cell counts were determined. The results of this experiment are shown in Table 4. This experiment shows that adequate gaseous exchange is required in order to maintain cell viability in alginate solutions.

TABLE 4

<u>Organism</u>	<u>Storage Tempera- ture</u>	<u>Log viable count/ML</u>				
		<u>T₀</u>	<u>1wk</u>	<u>2wks</u>	<u>3wks</u>	<u>5wks</u>
<u>P. fluorescens</u> 17-34	RT	12.03	9.79	5.32	2.71	3.79
	30°C	11.72	9.97	6.37	5.76	3.97
<u>P. putida</u> 1-104	RT	11.44	—	9.20	6.20	3.62
	30°C	11.45	—	5.32	4.32	3.86
<u>Pseudomonas sp.</u> 55-14	RT	11.80	11.64	5.70	4.72	3.97
	30°C	11.88	11.67	8.15	1.79	0
<u>Pseudomonas sp.</u> 67-4	RT	12.60	—	3.34	3.46	3.32
	30°C	12.84	—	3.46	3.75	3.62
<u>Bacillus sp.</u> 56-64	RT	9.32	9.46	9.17	5.72	5.35
	30°C	9.32	9.57	5.71	4.67	2.98
<u>P. putida</u> G2-8	RT	12.41	—	5.46	5.45	5.31
	30°C	12.45	—	5.62	4.25	3.32

Example 6: Survival of Rhizobium japonicum 138 in
Alginate Solutions and Oil Emulsions
in Bulk and When Coated onto Soyabeans

Rhizobium japonicum cultures were grown as described in Example 1. 250 ml of a fresh, healthy R. Japonicum culture was mixed with 250 ml of a sterile 1% non-crosslinked alginate solution. This mixture was designated solution A. 100 ml aliquots of solution A were removed and mixed with Food and Cosmetic Blue Dye #1 (final dye concentration = 1.0%) (to form mixture B) or with Food and Cosmetic Blue Dye #1 (final dye concentration = 1.0%) and Tween 81 (final concentration = 2.0%) (to form mixture C). The mixtures were i) added to the alginate prior to sterilization (blue dye) and ii) sterilized independently and added to the final mixture (Tween).

4 ml aliquots of the above mixtures were then dispensed into sterile 4.5 ml glass vials. In addition, 0.8 ml aliquots of solution C were dispensed into vials containing 3.2 ml of sterile mineral oil (to form solution D). All vials were then tightly sealed and stored at room temperature. Vials were removed periodically and agitated vigorously prior to sampling for viable cell count analysis. Table 5 shows the effect of time on the viability of cells in mixtures A-D.

Additionally, at the time of preparation 0.4ml samples of mixtures A-D were used to coat 10g samples of soybean seeds. Aliquots of coated seeds were removed periodically to allow the number of viable cells surviving on the seed coating to be determined. Figure 6 shows the effect of time on the viability of

cells of mixtures A-D coated onto soybeans. While viability of R. japonicum was maintained in all the mixtures, the alginate/cell/oil emulsion is preferred since this composition facilitated seed coating and enhanced the flowability of the seeds. Successful survival on the seed coat also shows that the mixtures can be employed as seed inoculants.

TABLE 5

	Log viable count/ml					
	<u>To</u>	<u>3 wks</u>	<u>7 wks</u>	<u>10 wks</u>	<u>16 wks</u>	<u>37 wks</u>
A	10.40	9.58	9.33	9.26	8.11	7.75
B	10.93	10.93	9.81	9.39	7.71	7.99
C	9.65	8.46	9.20	8.75	6.30	6.31
D	10.52	10.62	10.18	9.92	8.70	8.20

TABLE 6

	Log viable count/seed (stored at room temperature)					
	<u>To</u>	<u>2h</u>	<u>4h</u>	<u>6h</u>	<u>24h</u>	<u>1 wk</u>
A	6.22	5.87	5.55	4.62	4.64	3.27
B	6.61	6.35	6.43	4.66	5.24	3.65
C	6.01	5.47	5.12	4.81	4.48	2.73
D	5.39	5.66	5.35	4.25	5.24	3.79

Example 7: Effect of Polysaccharide on Stability
of Bacillus sp. 86-64

Bacillus sp. 86-64 was grown as described in Example 1. 50 ml aliquots of a fresh, healthy culture were mixed with an equal amount of a solution containing: 6% alginate; 4% alginate; 2% alginate; 4% alginate and 1.8% saline; 4% alginate and 1.0M sucrose; 4% alginate and 1% activated charcoal; 4% alginate and 1.5% cyclodextrin; 4% alginate and 2% corn starch; 4% alginate and 2% glycerol; 4% alginate and 1% gelatin; or 4% dextran, to form the following mixtures: Mixture A - culture medium + 3% alginate; Mixture B - culture medium + 2% alginate; Mixture C - culture medium + 1% alginate; Mixture D - culture medium + 2% alginate + 0.9% saline; Mixture E - culture medium + 2% alginate + 0.5M sucrose; Mixture F - culture medium + 2% alginate + 0.5% activated charcoal; Mixture G - culture medium + 2% alginate + 0.75% cyclodextrin; Mixture H - culture medium + 2% alginate + 1% corn starch; Mixture I - culture medium + 2% alginate + 1% glycerol; Mixture J - culture medium + 2% alginate + 0.5% gelatin; Mixture K - culture medium + 2% dextran. All alginate solutions contained only non-crosslinked alginates. The mixtures were stored at room temperature and sampled periodically to determine the viable cell count. The result, given in Table 7, shows that a variety of polysaccharide mixtures and alginate together with various adjuvants enhanced the stability of Bacillus sp. 86-64.

TABLE 7
Log viable count/ml

	<u>to</u>	<u>4 wks</u>	<u>10 wks</u>	<u>29 wks</u>
A	8.94	8.91	7.52	7.28
B	8.68	8.38	7.52	7.51
C	8.61	8.06	6.09	7.98
D	8.75	8.78	7.33	6.50
E	8.71	8.77	7.52	5.59
F	8.60	9.26	7.90	7.23
G	8.64	8.13	6.45	6.51
H	8.69	8.56	7.12	7.63
I	8.69	8.75	7.89	7.60
J	8.80	8.09	7.59	7.24
K	8.73	8.32	6.43	6.55

Example 8: Viability of P. fluorescens 17-34,
Pseudomonas sp. 55-14 and Bacillus sp.
84-64 in Alginate Mixtures and
Alginate/Oil Emulsions

50 ml aliquots of fresh healthy cultures of Pseu-
domonas fluorescens 17-34, Pseudomonas sp 55-14 and
Bacillus sp. 86-64 were grown as described in Example
1. Cultures were grown in 250ml Erlenmeyer flasks.
50ml of the culture broth were mixed with equal ali-
quots of the following non-crosslinked polysaccharide
compositions: (A) 4% alginate; (B) 4% alginate plus 2%
Food and Cosmetic Blue Dye #1; and (C) 4% alginate
plus 2% (final concentration) Food and Cosmetic Blue
Dye #1 plus 2% activated charcoal. Additionally, 10ml

culture aliquots were mixed with 10ml aliquots of the above alginate/cye mixture and either (D) 80ml sterile soya oil; (E) 78ml sterile soya oil plus 2ml Arlacel 83TM or (F) 78ml sterile soya oil plus 2ml Arlacel 186TM. All flasks were stoppered with cotton wool bungs and stored at room temperature. At various times, the mixtures were well agitated and a viable cell count determination was made.

The results, shown in table 8, indicates that incorporation of the alginate/cell solutions into an oil emulsion enhanced cell survival.

TABLE 8

Log viable count/ml

	<u>Bacillus sp. 86-64</u>			<u>Pseudomonas sp. 55-14</u>			<u>P. fluorescens 17-34</u>		
	<u>To</u>	<u>1mo</u>	<u>6mo</u>	<u>To</u>	<u>1mo</u>	<u>6mo</u>	<u>To</u>	<u>1mo</u>	<u>6mo</u>
A	7.16	7.99	8.00	9.96	8.10	7.23	11.05	7.81	8.01
B	7.86	8.10	7.35	11.33	8.80	7.41	11.22	8.34	7.97
C	7.62	8.32	6.71	11.06	8.61	7.68	11.04	8.35	7.93
D	8.78	11.33	11.81	8.35	11.92	10.96	8.53	11.94	11.57
E	8.51	8.33	12.04	8.25	11.32	11.36	8.57	11.69	11.62
F	8.46	—	9.76	8.47	12.40	9.51	8.41	11.25	11.31

Example 9: Survival of Rhizobacteria Coated onto Soyabeans and Canola (rape) Seed Using an Alginate/Oil Emulsion System

Cultures of rhizobacterial strains were prepared as described in Example 1

Fresh, healthy cultures were mixed with equal volumes of sterile 4% alginate. Aliquots of the culture/alginate solutions were then incorporated into emulsions with either canola or soya oil in a ratio of 30% aqueous: 68% oil: 2% emulsifier (Tween 81) to produce mixtures which were then coated onto canola seeds or soyabeans using 60 uL of mixture/10g of seed or 75uL of mixture/25g of beans. Seed samples were removed periodically to determine the viable cell count. The results, presented in Table 9, show the efficacy of the formulation as a microbial seed inoculant delivery system.

TABLE 9

		Log viable count/seed				
		<u>To</u>	<u>2h</u>	<u>24h</u>	<u>48h</u>	<u>96h</u>
Coated	<u>Azospirillum</u> sp.	2.94	2.58	1.98	2.14	-
onto	<u>Arthrobacter</u> sp. 44-9	2.55	2.55	2.22	2.35	-
canola	<u>P. fluorescens</u> 34-13	3.15	3.10	2.91	3.20	-
seeds in						
canola						
emulsion						
Coated	<u>Bacillus</u> sp. 86-64	5.31	4.92	4.56	-	3.81
onto	<u>Azospirillum</u> sp.	4.06	3.43	3.03	-	2.21
soyabeans	<u>P. putida</u> 57-10	4.95	4.41	3.95	-	3.33
in soya	<u>P. fluorescens</u> G20-18	4.31	3.95	3.43	-	3.38
oil	<u>Serratia liquifaciens</u> 1-102	3.93	3.75	3.02	-	2.16
emulsion						

Example 10: Survival of S. liquifaciens sp. 1-102
in Powder Produced by Drying Alginate/Culture Solution

Serratia liquifaciens 1-102 was grown as described in Example 1. An aliquot of fresh, healthy Serratia liquifaciens 1-102 culture was mixed with 1% sterile non-crosslinked alginate. The resultant mixture was then dried by blowing sterile, filtered air (30°C, liters/min) across the surface of 100 ml. aliquots of the mixture in 4L Erlenmyer flasks. The material obtained was then stored in a sealed container and sampled periodically for viable cell count determination. The results obtained, given in Table 10, show that this composition can be used to maintain viability of S. liquifaciens sp. 1-102.

TABLE 10

<u>To</u>	<u>Log viable count/g</u>			
	<u>3 days</u>	<u>9 days</u>	<u>15 days</u>	<u>60 days</u>
10.21	12.11	11.60	12.06	11.28

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for maintaining the viability of bacterial cells having a beneficial effect on plants
5 comprising maintaining a suspension of the bacterial cells in a free-flowing composition comprising a water dispersible, substantially soluble, non-crosslinked polysaccharide.
- 10 2. A method for inoculating a plant seed with bacterial cells having a beneficial effect on plants comprising contacting the plant seed with a free flowing composition which comprises (i) a suspension of the bacterial cells and (ii) a water dispersible,
15 substantially soluble, non-crosslinked polysaccharide.
3. A method for maintaining the viability of bacterial cells having a beneficial effect on plants comprising drying a free-flowing composition, the
20 composition comprising a suspension of the bacterial cells and a water dispersible, substantially soluble, non-crosslinked polysaccharide.
4. A method for inoculating a plant seed with
25 bacterial cells having a beneficial effect on plants comprising
 - (a) drying a free-flowing composition, the composition comprising a suspension of the bacterial cells and a water dispersible, substantially soluble,
30 non-crosslinked polysaccharide, and
 - (b) contacting the dried composition with the plant seed.
5. A method as claimed in any of claims 1 to 4
35 wherein the composition additionally contains an oil.



6. A method as claimed in any of claims 1 to 5 wherein the non-crosslinked polysaccharide is alginate.

7. A method as claimed in claim 6 wherein the alginate is present in the composition at a concentration of between 0.1-10%.

8. A method as claimed in any of claims 3 to 7 wherein the drying is accomplished by air drying at ambient temperature.

9. A composition capable of maintaining the viability of bacterial cells having a beneficial effect on plants comprising bacterial cells and a water dispersible substantially soluble non-crosslinked polysaccharide, the composition being a optionally dried free flowing suspension.

10. A plant seed composition capable of inoculating plant seed with bacterial cells having a beneficial effect on plants, the composition comprising plant seed, the bacterial cells and an optionally dried free flowing suspension of a water dispersible, substantially soluble non-crosslinked polysaccharide.

DATED this 29th day of April, 1991

Allelix Inc.

By Its Patent Attorneys

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