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(71) Applicant: **CHR. HANSEN A/S** [DK/DK]; Boege Alle 10-12, 2970 Hoersholm (DK).

(72) Inventors: **BANERJEE, Soumi**; C/O Chr. Hansen A/S, Boege Alle 10-12, 2970 Hoersholm (DK). **MOELBAK, Lars**; C/O Chr. Hansen A/S, Boege Alle 10-12, 2970 Hoersholm (DK).

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(54) Title: A MICROBIAL FORMULATION WITH REDUCED PRECIPITATION AND IMPROVED VISCOSITY

(57) Abstract: The present disclosure generally relates to compositions comprising Bacillus spp. with a reduced precipitation and an improved viscosity, to its use as a biological, to a process for its preparation, and to a kit. Particularly, the disclosure relates to compositions for agricultural use.

A MICROBIAL FORMULATION WITH REDUCED PRECIPITATION AND IMPROVED VISCOSITY

FIELD

The present disclosure generally relates to compositions comprising *Bacillus* spp. with a reduced precipitation and an improved viscosity, to its use as a biological, to a process for its preparation, and to a kit. Particularly, the disclosure relates to compositions for agricultural use.

BACKGROUND

As many chemical pesticides are being banned across the world, there is a growing market for microbial based biological agricultural formulations. For agriculture and plant health applications, some of the desired product formats containing microbial actives are wettable powders (WP), water dispersible granules (WDG) and liquid formulations, like suspension concentrates (SC). Especially the SC formulations have some intrinsic challenges because these involve suspending particles (such as spores) in the liquid, and these particles can sediment under gravity. On the other hand, in liquid formulations, which doesn't involve suspending particles such problems do not occur. In order to formulate biological actives such as *Bacillus* spores in suspension concentrates (SC), often co-formulants, such as, dispersants (surfactants), steric stabilizers (polymers), pH regulators (organic or inorganic acids), antifoams etc. are added to achieve good storage stabilities and desired functionalities during application. In addition to suspension stability of SC during shelf storage and transportation, good pourability and no/low residues upon dilution during application in field are highly desirable. For example, if there are significant number of residues of size greater than 75 µm present then it leads to applications challenges such as settling in mixing tanks and blocking of nozzles. Therefore, agricultural formulations are characterized by CIPAC (Collaborative International Pesticides Analytical Council Limited) methods such as MT185 (CIPAC Handbook K) to characterize the residues.

While the pourability of a formulation depends on the viscosity, which in turn depends on ingredients selected and the amounts used, the residue in a formulation depends on the solubility of the ingredients. The solubility of the ingredients in turn depends on the type of water used for preparing the SC, as well as the type of water used for dilution of SC during the field application. An important parameter related to water quality is its

hardness. Hardness of water originate from the presence of the multivalent cations. Out of the many cations that can be present in water, Calcium (Ca^{2+}) and Magnesium (Mg^{2+}) are the two most abundantly available multivalent cations that pose threats to the formulation efficacy. Ca^{2+} and Mg^{2+} can bind strongly to the co-formulants usually present in a suspension concentrate mentioned above and can render those ineffective by precipitating them out and thereby making the dispersion unstable. Another problem is the possible formation of undesired residues of larger particle size giving problems with blocking of nozzles and settling in mixing tanks. These problems will become even more important in formulations using tap water for mixing, as tap water is bound to contain a certain degree of hardness. Further, during application, the dilution of SC typically takes place using locally available water in the field and depending on the region the water can have various degrees of hardness.

In order to address the problems faced with agricultural formulations and provide a solution, the inventors investigated the effect of using chelating agents such as EDTA as an additive in microbial formulations comprising *Bacillus* spp. for use as an agricultural formulation. Precipitation is a commonly known problem when working with bacterial formulations and trying to solve this problem has been attempted in the past.

WO2022/003113 discloses a method of reducing precipitation in a bacterial fermentation process and a media system for use in a bacterial fermentation process, said media comprising an amount of calcium salts, magnesium salts, phosphate salts and at least one chelating agent, which is a citrate salt and/or citric acid at an amount of at least 5 mM, and/or EDTA at an amount of at least 1 mM. The problem solved by the described invention is a problem with precipitation in a bacterial fermentation process and does not relate to agricultural formulations. Thus, there is no mention or description of the problems associated with precipitation in agricultural formulations and the need for a reduction of that while also improving viscosity.

US20200131096 discloses the possibility of adding a chelator to a biofertilizer composition. Several long lists of different chelated metals and organic acid chelating agents are present in the document and there is no clear and unambiguous teaching of the actual use of chelating agents. In fact, the biofertilizer compositions described in the example does not comprise chelated metals or organic acid chelating agents and there is no description of reduced precipitation and improved viscosity of the described composition. The opposite is in fact the case as it is clearly described that residues are formed and that they are removed by rinsing the tank.

Thus, there is still a need for novel agricultural formulation composition including a SC formulation with reduced precipitation and improved viscosity even after using local tap water for dilution of the SC formulation.

The inventors wanted with their agricultural formulation to ensure the efficacy of the formulated product in hard water and to avoid any deteriorating effect caused by the precipitates (residues) formed in the hard water. Furthermore, the addition of EDTA was surprisingly found to also modify the rheological properties of the SC, e.g. the viscosity was found to be significantly improved.

SUMMARY

The present disclosure provides an agricultural formulation composition for use as a biological product for agricultural application which solve the problem of providing biological compositions comprising microorganisms for agricultural use, which show reduced precipitation and improved viscosity. Also disclosed is a process for preparing the composition and use of the composition as a biostimulant, bionematicide, biofungicide, or biofertilizer.

Solution Concentrate (SC) agricultural formulations contain ingredients such as anionic surfactants and citric acid which can form calcium salts and these insoluble salts can precipitate leading to residues (crystals) of size > 75 µm which are highly undesirable during application. Residues of that size will lead to sedimentation in the tank and will lead to blocking of the nozzles of the machines used to spread the SC agricultural formulation in the field.

By using a chelating agent in the agricultural formulations, this chelating agent will bind calcium and magnesium ions and form soluble salts that will not precipitate out of solution. Thus, the colloidally stable complexes that are formed will not cause any challenges during application in the field. In addition, the viscosity of the SC agricultural formulations is significantly modified and improved by use of chelating agents in the agricultural formulation.

The purpose of the studies described in the examples was to investigate the effect of different composition on the precipitation and on the viscosity of the composition. Both parameters are essential features to optimize in order to provide a good liquid formulation for agricultural use.

The examples describe and demonstrate the benefits of the composition, said composition having reduced amount of precipitation and an improved viscosity at the same time.

DEFINITIONS

In general, the terms and phrases used herein have their art-recognized meaning, which can be found by reference to standard textbooks, journal references and context known to those skilled in the art. The following definitions are provided to clarify their specific use in context of the present disclosure.

As used herein, the singular forms "a", "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

As used herein, the term "and/or" is intended to mean the combined ("and") and the exclusive ("or") use, i.e. "A and/or B" is intended to mean "A alone, or B alone, or A and B together".

Co-solvent: As used herein the term "co-solvent" refers to a liquid miscible with water, such as glycerol, propylene glycol.

Composition: As used herein the term "composition" refers to a composition comprising a suspension of biological actives, such as at least one bacterial strain as described herein in a liquid such as water wherein other ingredients are added to the liquid to physically stabilize the bioactive and they are part of the composition.

Dispersant: As used herein the term "dispersant" refers to low molar mass or polymeric materials that can stabilize particles in liquid and help them suspend (disperse) well in the continuous liquid phase. Examples of dispersants are ethoxylated nonyl phenols, maleic anhydride copolymers, linear alkyl benzene sulphonates, lignosulfonates, sodium dodecylsulphate, polyalkoxylated alcohol, tridecanol ethoxylates naphthalene sulphonate formaldehyde condensates, polysorbates, sodium polynapthalene sulphonate, polymethyl methacrylate-polyethylene glycol graft copolymers, salts of ethoxylated phosphate esters, butyl polyalkylene oxide block copolymers and condensed phenolsulphonic acid and their salts

Effective amount/concentration/dosage: As used herein the terms "effective amount", "effective concentration", or "effective dosage" are defined as the amount, concentration, or dosage of the bacterial strain(s) and ingredients sufficient to reduce the precipitation and improve the viscosity of the agricultural composition and confer benefits similar to the ones demonstrated in the examples.

The actual effective dosage in absolute numbers depends on factors including: the hardness of the water in question, other ingredients present. The "effective amount", "effective concentration", or "effective dosage" of the bacterial strains and other ingredients may be determined by routine assays known to those skilled in the art. An example of an effective amount is given in Examples 2, 3 and 4.

Isolated: As used herein the term "isolated" means that the bacterial strains described herein are in a form or environment which does not occur in nature, i.e. the strain is at least partially removed from one or more or all of the naturally occurring constituents with which it is associated in nature.

Biological product for agricultural application: As used herein the term "biological product for agricultural application" means a product which contains as an active ingredient one or more bacterial strains. The biological product for agricultural application may have different functionalities and depending on that can be used as a biostimulant, bionematicide, biofungicide, or biofertilizer.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1

Figure 1 discloses a picture of residues left after wet sieve analysis (CIPAC method MT 185) of SC1 and SC1 + EDTA.

FIGURE 2

Figure 2 discloses characterization of residues obtained after wet sieve analysis of SC. **(a)** Stereo microscope image of the residue obtained after the wet sieve test of the SC. The SC was diluted in tap water of certain hardness. The horizontal red bar represents the magnification of the image. **(b)** SEM image of calcium citrate residue. The image is acquired at 1000 times magnification. The horizontal bar represents the magnification of the image. **(c)** EDX spectrum of calcium citrate residue. The EDX-spectrum shows that the sample contains carbon (C), calcium (Ca), oxygen (O), and small amounts of chlorine (Cl), sulphur (S), sodium (Na), silicon (Si), potassium (K), copper (Cu), and phosphorus (P). **(d)** Powder X-ray diffraction pattern of calcium citrate residue between 5 and 85° 2θ. **(e)** Line plot between 5 and 45 °2θ showing the positions of reflections in the raw data (orange) and the line positions in the reference phases calcium citrate tetrahydrate (blue) and sodium chloride (green). ID numbers for PDF-4+ reference patterns are sodium chloride (NaCl): 01-077-3565 and calcium citrate tetrahydrate ($Ca_3(C_6H_5O_7)_2 \times 4H_2O$): 00-069-1272. The black arrows show the positions of lines belonging to unidentified phase(s). **(f)** Chemical formula of calcium citrate tetrahydrate, $Ca_3(C_6H_5O_7)_2 \times 4H_2O$. The citric acid has three carboxylic acid groups with pKa of 3.13, 4.76 and 6.40. **(g)** Calcium citrate crystals in pure water are insoluble. **(h)** Calcium citrate crystals got dissolved in 0.1 M Dissolvine in water.

FIGURE 3

Figure 3 discloses viscosity of different suspension concentrate (SC) formulations with or without EDTA added to the SCs.

DETAILED DESCRIPTION

Rhizobacteria

The soils are home to a complex biological community, of which micro-organisms, prokaryotes and eukaryotes form a majority, both in number and in diversity. Some prokaryotes have ecological niches as the rhizosphere, and/or the rhizoplane of plants, where they multiply, survive and protect themselves from the antagonistic action of soil microflora. These organisms have been generically called rhizobacteria.

In association with plants, rhizobacteria may have a deleterious effect, null or beneficial. Those who exercise a beneficial effect - growth promotion and biological control of disease - are called PGPR ("Plant Growth-Promoting Rhizobacteria). It is estimated that only 0.6 % of rhizobacteria have some beneficial effect for the plant with which they are associated.

PGPR have been used for biological control of plant diseases and thereby increase the productivity of crops. How and why this biological control is exercised, is still a topic that needs complementary studies.

Agricultural formulations comprising microorganisms including rhizobacteria have been used for many years. There is a continued need for optimizing these agricultural formulation compositions and solving the inherent problems with precipitation and optimization of viscosity.

Agricultural formulation composition

The agricultural formulation composition of the present disclosure incorporates microorganisms preferably spore-forming rhizobacteria into an agricultural formulation composition with reduced precipitation problems and with an improved viscosity.

In one embodiment of the disclosure an agricultural formulation composition comprises as an active ingredient a microorganism as also described herein below in the section "Microorganisms for agricultural formulation composition".

Also present in such an agricultural formulation composition is different types of dispersants. The dispersant is an agent for improving dispersibility and prevents agglomeration between particles. In the present disclosure the dispersants used in the agricultural formulation may be an anionic dispersant and/or a non-ionic dispersant. Examples of anionic dispersants include, but are not limited to linear alkyl benzene sulphonates, lignosulfonates, sodium dodecylsulphate, naphthalene sulphonate formaldehyde condensates, sodium polynapthalene sulphonate, salts of ethoxylated phosphate esters and condensed phenolsulphonic acid and their salts.

Examples of non-ionic dispersants include, but are not limited to ethoxylated nonyl phenols, polyalkoxylated alcohol, and polysorbates.

In an agricultural formulation composition within the present disclosure the amount of dispersant will be evaluated in relation to the other ingredients present in the agricultural formulation composition. In one embodiment of the present disclosure the dispersant constitutes 0.001 – 25 % (w/w) of the total composition, such as 0.05-15% (w/w), such as 0.5-10% (w/w), such as 2-5% (w/w), such as 2-3% (w/w). In one embodiment of the present disclosure the dispersant constitutes 2.8 % (w/w) of the final formulation.

In addition the agricultural formulation composition of the present disclosure may include a stabilizer. Such a stabilizer is in one embodiment a polymeric stabilizer. Examples of polymeric stabilizers include, but are not limited to maleic anhydride copolymers, polymethyl methacrylate-polyethylene glycol graft copolymers, and butyl polyalkylene oxide block copolymers.

In an agricultural formulation composition within the present disclosure the amount of stabilizer will be evaluated in relation to the other ingredients present in the agricultural formulation composition. In one embodiment of the present disclosure the stabilizer constitutes 0.01-10% (w/w) of the final formulation, such as 0.02-8% (w/w), such as 0.25-5% (w/w), such as 0.25-1.5% (w/w), such as 0.4-1% (w/w) of the final formulation. In one embodiment of the present disclosure the stabilizer constitutes 0.5 % (w/w) of the final formulation.

In one embodiment of the present disclosure the agricultural formulation composition may comprises an antifoam agent. Examples of antifoam agents include, but are not limited to, Silfoam 150, SAG 1572, OR-10, and SB509.

In an agricultural formulation composition within the present disclosure the amount of antifoam agent will be evaluated in relation to the other ingredients present in the agricultural formulation composition. In one embodiment of the present disclosure the antifoam agent constitutes 0.002-2% (w/w) of the final formulation, such as 0.01-1% (w/w), such as 0.05-0.3% (w/w), such as 0.08-0.15% (w/w) of the final formulation. In one embodiment of the present disclosure the antifoam agent constitutes 0.1 % (w/w) of the final formulation.

A skilled person working with agricultural formulation compositions will know that different types of salts may be added to the agricultural formulation composition in order to regulate the osmolarity of the final formulation. Examples of such salts include, but are not limited to sodium chloride, potassium chloride, and sodium nitrate. In one embodiment of the present disclosure the salt constitutes 0.25-3% (w/w) of the final formulation, such as 0.5-2% (w/w). In one embodiment of the present disclosure the salt constitutes 1% (w/w) of the final formulation.

The adjustment of pH might also be necessary and it is therefore within the present disclosure to optionally include pH regulators in the agricultural formulation composition. Examples of such pH regulators include, but are not limited to hydrochloric acid, phosphoric acid, citric acid, acetic acid, maleic acid, and lactic acid. In one embodiment of the present disclosure the pH regulator constitutes 0.05-6% (w/w) of the final formulation, such as 0.1-3% (w/w), such as 0.25-2% (w/w), such as 0.5-1.25% (w/w) of the final formulation. In one embodiment of the present disclosure the pH regulator constitutes 0.94% (w/w) of the final formulation.

In one embodiment of the present disclosure the agricultural formulation composition comprises a co-solvent. Examples of co-solvent relevant for the present disclosure includes, but are not limited to glycerol, propylene glycol, and ethylene glycol. In one embodiment of the present disclosure the co-solvent constitutes 1 – 50 % (w/w) of the total composition, such as 2-30% (w/w), such as 5-15% (w/w), such as 8-12% (w/w).

In one embodiment of the present disclosure the co-solvent constitutes 10% (w/w) of the final formulation.

The agricultural formulation composition of the present disclosure is in the form of a solution concentrate (SC). In a solution concentrate water constitute an essential part of the formulation and acts as a carrier. In an agricultural formulation composition within the present disclosure water constitutes 30 – 98.874 % (w/w) of the total composition, such as 40-75% (w/w), such as 50-70% (w/w). In one embodiment of the present disclosure water constitutes 53-60% (w/w) of the final formulation.

It is within the present disclosure that the agricultural formulation composition is further diluted on-site by the end user, using local tap water for further dilution of the agricultural formulation composition. The agricultural formulation composition of the present disclosure may be diluted 8-12 times by the end user, such as 9-11 times. In one embodiment of the present disclosure the agricultural formulation composition is diluted 10 times by the end user.

The water intended to be used in the disclosed agricultural formulation composition may have differing hardness depending. The hardness of the water may be in the range of 50 – 1500 ppm.

One essential ingredient in the agricultural formulation compositions of the present disclosure is the chelating agent. As also described in the summary, the present disclosure describes the realization made that by using a chelating agent in the agricultural formulation compositions, this chelating agent will bind calcium and magnesium ions and form soluble salts that will not precipitate out of solution. Thus, the colloidally stable complexes that are formed will not cause any challenges during application in the field. In addition, the viscosity of the SC agricultural formulations is significantly modified and improved by use of chelating agents in the agricultural formulation composition. Examples of chelating agents useful in the present disclosure includes, but are not limited to sodium-EDTA, potassium-EDTA, fulvic acid, humic acid, propionic acid, monovalent salts of citric acid such as trisodium citrate, homo citric acid, ascorbic acid, lactic acid, oxalic acid, vanillic acid, gallic acid, malate, and gluconic acid,

monovalent salts of phosphoric acid such as trisodium phosphate, or organic acid such as citric acid and the monovalent metal salt thereof.

A chelating agent such as sodium-EDTA will form strong soluble complex with Ca2+ and/ or Mg2+, which are present in water, and will protect the co-formulants from depletion from the bulk of the formulation. Ca-EDTA or Mg-EDTA complexes remain soluble in water and hence, formation of residues can be completely avoided, and the efficacy of the formulations can be maintained even in hard water (i.e. water high in dissolved minerals, largely calcium and magnesium). Such an approach has been used extensively in other industries such as laundry detergent formulations, however, it seems that not only has this concept not been tried extensively in the field of microbial formulations for agricultural applications, but never has in the past been seen an improvement in rheological behavior (viscosity modification) of SCs due to the incorporation of a chelating agent.

In one embodiment of the present disclosure the agricultural formulation composition the chelating agent or monovalent salt thereof constitutes 0.025 – 10 % (w/w) of the total composition, such as 0.05-7% (w/w), such as 0.1 – 3% (w/w), such as 0.3-1.5% (w/w). In one embodiment of the present disclosure the chelating agent or monovalent salt thereof constitutes 0.5% (w/w) of the final formulation.

Examples of agricultural formulation compositions within the present disclosure is provided in the Examples section herein below.

Microorganisms for agricultural formulation compositions

The present disclosure describes an agricultural formulation composition comprising at least one microorganism, at least one chelating agent and water.

Agricultural formulation compositions useful in this regard are described in more detail in the above section "Agricultural formulation compositions". The microorganisms may include species of bacteria and fungi, including yeast and mold species. Suitable microorganisms include those commonly known in the art as phosphorus-solubilizing, potassium-solubilizing, nitrogen-fixing (collectively, biofertilizer), phototrophic, lactic acid, and sulfide-utilizing microorganisms.

It is contemplated that where two or more microorganisms are present within one or more embodiments of the composition, the microorganisms may be co-cultured. The microorganisms may be propagated by methods known in the art. For example, the microorganisms may be propagated in a liquid medium under anaerobic or aerobic conditions. Suitable liquid mediums used for growing microorganisms include those known in the art. It is contemplated that, in some embodiments, the one or more strains of useful microorganisms described herein may be co-cultured under laboratory conditions in any combination before being transferred to one or more fermentation tanks. In some embodiments, the one or more strains of useful microorganisms may be co-cultured partly under laboratory conditions, then transferred to one or more fermentation tanks, where the co-culture process may continue. In other embodiments, the one or more strains of useful microorganisms may be co-cultured within one or more fermentation tanks.

In one embodiment, the microorganism consortium of the agricultural formulation composition includes live microorganisms. In one embodiment, at least one microorganism is included in the microorganism consortium of the disclosure. In another embodiment, the compositions include consortia of two or more microorganisms. In another embodiment, the microorganism consortium of the agricultural composition includes living and non-living microorganisms. Compositions containing non-living microorganisms may contain extracts of the microorganisms. Such extracts may be considered a liquid fermentation product of the living microorganisms. The extracts of microorganisms include, by way of example, enzymes, metabolites, proteins, and other substances that are produced by microorganisms and are capable of eliciting an effect on an environment regardless of the living status and/or metabolic states of the microorganism.

In one embodiment, the microorganism composition may be fermented, and may therein produce one or more fermentation products. The microorganism composition may be fermented for about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more days. In one embodiment, the microorganism composition is fermented for at least about 15 to about 23 days. In another embodiment,

the microorganism composition is fermented for at least 21 days. In another embodiment, the microorganism composition is fermented for at least 15 days.

In one embodiment, the process of fermentation may result in one or more fermentation products. The one or more fermentation products may comprise an organic acid, an alcohol, or another type of compound.

In one embodiment, the microorganism composition includes a total number of microorganisms of about 1 to about 1 million colony forming units (CFU) per milliliter. In another embodiment, the microorganism composition includes a total number of microorganisms of about 100,000 to about 800,000 CFU per milliliter. In yet another embodiment, the microorganism composition includes a total number of microorganisms of about 250,000 to about 600,000 CFU per milliliter. In an additional embodiment, the microorganism composition includes a total number of microorganisms of about 300,000 CFU per milliliter.

The agricultural formulation composition of the present disclosure includes from 10^5 to 10^{12} CFU/ml in the final formulation.

In one embodiment of the present disclosure the microorganism composition or consortium of microorganisms constitutes 0.05-60% (w/w) of the final composition, such as 0.1 – 50 % (w/w), such as 5-30% (w/w) of the total composition. In one embodiment of the present disclosure the microorganism composition or consortium of microorganism constitutes 25-27% (w/w) of the final composition.

The spore-forming rhizobacteria have a number of advantages over chemical pesticides or even on other biological control agents: they are easy to mass-produce, they are easy to store, they are adaptable to the formulation technology and require no genetic manipulation.

The spore-forming rhizobacteria can be applied by treating the substrate, immersing the seedling root systems in bacterial suspensions, watering the plant with bacterial suspension, by dipping the seeds in suspension of rhizobacteria or by applying PGPR with the pelleting of seeds.

One example of a spore-forming rhizobacteria is *Bacillus* spp. which have drawn significant attention in recent years because of their safety to the environment and ability to deliver different modes of action for suppression of nematode population in the soil.

The *Bacillus* species are Gram-positive bacteria characterized by having thick cell walls and the absence of outer membranes, which differs from the Gram-negative bacteria. Much of the walls of Gram-positive bacteria is composed of peptidoglycan.

Gram-positive species are divided into groups according to their morphological and biochemical characteristics. The genus *Bacillus* is belonging to the group of Gram-positive spore-forming bacteria. Species forming spore structures that are resistant to environmental changes, sustain dry heat and certain chemical disinfectants for moderate periods of time. They persist for years on dry land.

The beneficial effect of *Bacilli*, such as e.g. *B. subtilis*, when applied near the seed or the soil, is not solely due to the antagonism afforded to pathogens. The PGPR has a positive influence on germination, development and crop yield due to the production of substances which promote plant growth (e.g. volatile organic compounds, phytohormones) and improvement in plant nutrition (e.g. solubilization of phosphorus).

The agricultural formulation composition of the present disclosure is useful for any application that involves a liquid formulation, for example the SC formulation.

Liquid formulations may be applied to the seed before sowing but are more often used for administration to the field either in furrow, drip applied to the soil or spray on the foliage of the crop.

The purpose of the administration of the agricultural composition depends on the ingredients in the agricultural composition and especially it depends on the microorganisms present in the composition. Different microorganisms have different effects and hence the agricultural composition may be used as a biostimulant (stimulating growth of the crop), as a biofungicide (preventing and/or treating fungal infections of the crop), or as a bionematicide (preventing and/or treating infections with nematodes of the crop).

In one embodiment of the present disclosure the microorganism consortium comprises at least one species selected from gram positive spore forming bacilli, such as, *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus paralicheniformis*, *Bacillus megaterium*, *Bacillus amyloliquefaciens*, and *Bacillus licheniformis.*

In another embodiment the microorganism consortium also comprises at least one species from the genus *Pristia* such as, *Pristia megaterium.*

The microorganism composition of the present disclosure may, besides the active microorganism components, contain agrochemical acceptable excipients, vehicles and/or adjuvants.

Agrochemically acceptable carriers, vehicles and/or adjuvants are considered to be known to the skilled reader and can be selected from the group consisting of, but not limited to maltodextrine, silicon dioxide, modified zeolite, kaolinite, lignin, starch, chitosan, and calcium carbonate. In a preferred embodiment, the agrochemically acceptable carriers, vehicles and/or adjuvants is maltodextrine and silicon dioxide.

In one embodiment, the microorganism composition of the present disclosure can be mixed with further ingredients relevant in the agrochemical field, including but not limited to a microbial, a biological, and/or a chemical insecticide, fungicide, nematicide, bactericide, herbicide, plant extract, plant growth regulator, and/or fertilizer, present in an amount suitable to benefit plant growth and/or to confer protection against a pathogenic infection in a susceptible plant, a carrier, a surfactant, a dispersant, enzyme(s) and/or a yeast extract.

Among the main crops of plants are sugar cane, coffee, soybeans, cotton, corn, potatoes, tomatoes, tobacco, banana, rice, wheat, barley, oilseed rape, avocado, pineapple, squash, cacao, coconut, oats, onion, lettuce, beet, carrot, cassava, beans, sunflower, pepper, turnip, apple, strawberry, okra, radish and onion.

In one embodiment the agricultural formulation composition is used in fruitculture, where citrus, grape, guava, papaya, fig, peach, plum and nespeira are of particular relevance and in horticulture, where eggplant and cruciferous are of particular relevance.

The agricultural formulation composition of the present disclosure may in one embodiment be applied to the fruit and/or vegetable after harvest.

In one embodiment the agricultural formulation composition is used in floriculture where rose, chrysanthemum, lisianthus, gerbera, amaryllis, begonia and celosia are of particular relevance.

In one embodiment of the present disclosure, the agricultural formulation composition is applied to seeds as part of a seed coating process.

The plant seed can include, but is not limited to, the seed of monocots and dicots, such as the seed of Cereals, Corn, Sweet Corn, Popcorn, Seed Corn, Silage Corn, Field Corn, Rice, Wheat, Barley, Sorghum, Brassica Vegetables, Broccoli, Cabbage, Cauliflower, Brussels Sprouts, Collards, Kale, Mustard Greens, Kohlrabi, Bulb Vegetables, Onion, Garlic, Shallots, Fruiting Vegetables, Pepper, Tomato, Eggplant, Ground Cherry, Tomatillo, Okra, Grape, Herbs/Spices, Cucurbit Vegetables, Cucumber, Cantaloupe, Melon, Muskmelon, Squash, Watermelon, Pumpkin, Eggplant, Leafy Vegetables, Lettuce, Celery, Spinach, Parsley, Radicchio, Legumes/Vegetables (succulent and dried beans and peas), Beans, Green beans, Snap beans, Shell beans, Soybeans, Dry Beans, Garbanzo beans, Lima beans, Peas, Chick peas, Split peas, Lentils, Oil Seed Crops, Canola, Castor, Cotton, Flax, Peanut, Rapeseed, Safflower, Sesame, Sunflower, Soybean, Root/Tuber and Corm Vegetables, Carrot, Potato, Sweet Potato, Beets, Ginger, Horseradish, Radish, Ginseng, Turnip, sugarcane, sugarbeet, Grass, or Turf grass.

In one or more embodiments, the plant seed can include seed of a drybean, a corn, a wheat, a soybean, a canola, a rice, a cotton, a grass, and a turf grass.

In an alternative embodiment, the agricultural formulation composition of the present disclosure comprising microorganisms, may be added to soil or growth medium surrounding the plant; soil or growth medium before sowing seed of the plant in the soil or growth medium; or soil or growth medium before planting the plant, the plant cutting, the plant graft, or the plant callus tissue in the soil or growth medium.

When the agricultural formulation composition of the present disclosure is added to soil or growth medium surrounding the plant; soil or growth medium before sowing seed of the plant in the soil or growth medium; or soil or growth medium before planting the plant, the plant cutting, the plant graft, or the plant callus tissue in the soil or growth medium, the agricultural formulation composition can include an amount of *Bacillus*,

from about 1.0 x 10^5 CFU/ml to about 1.0 x 10^{12} CFU/ml.

In one or more embodiments, the plant, the plant cutting, the plant graft, or the plant callus tissue can include soybean, bean, snap bean, wheat, cotton, corn, pepper, tomato, potato, cassava, grape, strawberry, banana, peanut, squash, pumpkin, eggplant, sugarcane and cucumber.

The agricultural formulation composition disclosed has the technical advantage that it has a reduced amount of residues and an improved viscosity making it an agricultural formulation composition particularly useful for application by spray. It is therefore useful in applications that includes spraying of the soil, spray coating the seed, spraying the fruit and/or vegetables.

In another embodiment, the agricultural formulation composition may be applied directly to the plant or part of the plant. Application may be accomplished by spraying the composition onto the foliage of the plants. This type of application may be referred to as foliar application and can typically be implemented in most agricultural settings without the need for investment in additional equipment.

Thus, an embodiment of the present disclosure relates to the use as described herein, wherein the composition is applied to one or more aerial surfaces of the plant.

Another embodiment of the present disclosure relates to the use as described herein, wherein the aerial surfaces of the plant are selected from the group consisting of leaves, stems, flowers, fruits or combinations thereof.

A further embodiment of the present disclosure relates to the use as described herein, wherein the composition is applied to the plant by spraying.

The agricultural formulations composition including the microorganism composition as described herein can be in the form of a liquid, a water-based dispersion. More specifically the composition may for example be an emulsion concentrate (EC), a suspension concentrate (SC), a suspo-emulsion (SE), a capsule suspension (CS), a water in oil emulsion (EO), an oil in water emulsion (EW), a dispersible concentrate (DC), or any technically feasible formulation in combination with agriculturally acceptable adjuvants.

The disclosure also relates to a process for preparing the agricultural formulation composition, as described herein above, comprising mixing, in desired ratios, the

mentioned ingredients. Mixing an agricultural formulation composition is well within the skills of a skilled person in the field of agricultural compositions.

The disclosure in one embodiment also relates to use of the agricultural formulation composition, as described herein above, as a biological product for agricultural applications on a plant culture. The biological product may be a biostimulant, bionematicide, biofungicide, and/or biofertilizer.

Embodiments and features of the present disclosure are also outlined in the following items.

Items

1. An agricultural formulation composition for use as a biological product for agricultural application comprising:
   a) a microorganism composition or a consortium of microorganisms in culture medium;
   b) at least one dispersant;
   c) at least one chelating agent or monovalent metal salt thereof;
   and
   d) water

   said composition having a precipitation of between 0.1% and 2% of wet-sieve residues when measured using the CIPAC MT185 method and a viscosity of 10 – 500 cP, when measured on a rotational viscometer at room temperature.

2. The composition according to item 1, wherein the precipitation is between 0.1% and 0.5% of wet-sieve residues when measured using the CIPAC MT185 method.

3. The composition according to any preceding items, wherein said microorganism composition or consortium of microorganisms comprises at least one species selected from the group consisting of Gram-positive spore formers such as *Bacillus* spp., such as *Bacillus megaterium*, *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus paralicheniformis*, *Bacillus amyloliquefaciens*, and *Bacillus licheniformis*.

4. The composition according to any preceding items, wherein said at least one dispersant is selected from the group consisting of: an anionic dispersant and/or

a non-ionic dispersant, such as linear alkyl benzene sulphonates, lignosulfonates, sodium dodecylsulphate, naphthalene sulphonate formaldehyde condensates, sodium polynapthalene sulphonate, salts of ethoxylated phosphate esters, condensed phenolsulphonic acid and their salts, ethoxylated nonyl phenols, polyalkoxylated alcohol, and polysorbates.

5. The composition according to any preceding items, wherein said at least one chelating agent is selected from the group consisting of Ethylenediaminetetraacetic acid (EDTA), Tetrasodium glutamate diacetate (GLDA) , methylglycine diacetic acid (MGDA), ethanoldiglycinic acid (EDG), Diethylenetriaminepentaacetic acid (DTPA), Hydroxyethylethylenediaminetriacetic acid (HEDTA) or any monovalent salt thereof.

6. The composition according to any preceding items, wherein said monovalent salt of said chelating agent is selected from the group consisting of: sodium-EDTA, potassium-EDTA, fulvic acid, humic acid, propionic acid, monovalent salts of citric acid such as trisodium citrate, homo citric acid, ascorbic acid, lactic acid, oxalic acid, vanillic acid, gallic acid, malate, and gluconic acid, monovalent salts of phosphoric acid such as trisodium phosphate, or organic acid such as citric acid and the monovalent metal salt thereof.

7. The composition according to any preceding items, further comprising a co-solvent selected from the group consisting of glycerol, and propylene glycol.

8. The composition according to any preceding items, further comprising a stabilizer selected from the group consisting of maleic anhydride copolymers, polymethyl methacrylate-polyethylene glycol graft copolymers, and butyl polyalkylene oxide block copolymers.

9. The composition according to item 8, wherein the stabilizer constitutes 0.25-1.5% (w/w) of the final formulation, such as 0.4-1% (w/w), such as 0.5 % (w/w) of the final formulation.

10. The composition according to any preceding item, wherein said composition further comprises an antifoam agent, such as Silfoam 150, SAG 1572, OR-10, and/or SB509.

11. The composition according to item 10, wherein the antifoam agent constitutes 0.05-0.3% (w/w) of the final formulation, such as 0.08-0.15% (w/w), such as 0.1 % (w/w) of the final formulation.

12. The composition according to any preceding item, wherein said composition further comprises a salt selected from the group consisting of sodium chloride, potassium chloride, and/or sodium nitrate.

13. The composition according to item 12, wherein the salt constitutes 0.25-3% (w/w) of the final formulation, such as 0.5-2% (w/w), such as 1% (w/w) of the final formulation.

14. The composition according to any preceding item, wherein said composition further comprises a pH regulator selected from the group consisting of hydrochloric acid, phosphoric acid, citric acid, acetic acid, maleic acid, and/or lactic acid.

15. The composition according to item 14, wherein said pH regulator constitutes 0.25-2% (w/w) of the final formulation, such as 0.5-1.25% (w/w), such as 0.94% (w/w) of the final formulation.

16. The composition according to any preceding items, wherein said water has a hardness of 50 – 1500 ppm.

17. The composition according to any preceding items, wherein said microorganism or consortium of microorganisms is present at 0.1 – 50 % (w/w) in the total composition.

18. The composition according to any preceding items, wherein said dispersant is present at 0.001 – 25 % (w/w) in the total composition.

19. The composition according to any preceding items, wherein said chelating agent or monovalent salt thereof is present at 0.025 – 10 % (w/w) in the total composition.

20. The composition according to item 7-19, wherein said co-solvent is present at 1 – 50 % (w/w) in the total composition.

21. The composition according to any preceding items, wherein said water is present at 30 – 98.874 % (w/w) in the total composition.

22. The composition according to any preceding items, wherein said composition is in the form of a liquid formulation.

23. The composition according to item 22, wherein additional water is added to the composition on-site by the user.

24. The composition according to item 23, where the liquid formulation is diluted 8-12 times by the additional water added on-site by the user.

25. The composition according to any preceding items, wherein said composition comprises from 1.0 x 10^6 CFU/gram to 1.0 x 10^9 CFU/gram of formulation.

26. Process for preparing the composition, as defined in any one of items 1 to 25, comprising mixing, in desired ratios, the mentioned ingredients.

27. Use of the composition, according to any one of items 1 to 25, or obtainable from a process as defined in item 26, as a biological product for agricultural applications on a plant culture.

28. Use of the composition according to item 27, as a biostimulant, bionematicide, biofungicide, or biofertilizer.

29. Use of the composition according to items 27-28, for preventing, controlling, combating and/or conferring induction of resistance to phytonematodes.

30. Use according to item 29, wherein the phytonematodes are selected from the group consisting of *Meloidogyne, Pratylenchus, Heterodera, Globodera, Ditylenchus, Tylenchulus, Xiphinema, Bursaphelenchus, Radopholus, Rotylenchulus, Helicotylenchus, Nacobbus, Aphelenchoides and Belonolaimus*.

31. The use according to item 27, for preventing, controlling, combating and/or conferring induction of resistance to phytopathogens, such as a plant fungal pathogen or a plant bacterial pathogen.

32. The use according to item 31, wherein said one or more phytopathogen(s) is from a genus selected from the group consisting of Fusarium, Botrytis, Erwinia, Dickeya, Agrobacterium, Xanthomonas, Xylella, Candidatus, Sclerotinia, Cercospora/Cercosporidium, Uncinula, Podosphaera, Phomopsis, Alternaria, Pseudomonas, Phytophthora, Phakopsora, Aspergillus, Uromyces, Cladosporium, Rhizopus, Penicillium, Rhizoctonia, Macrophomina, Mycosphaerella, Magnaporthe, Monilinia, Colletotrichum, Diaporthe, Corynespora, Gymnosporangium, Schizothyrium, Gloeodes, Botryosphaeria, Neofabraea, Wilsonomyces, Sphaerotheca, Erysiphe, Stagonospora, Pythium, Venturia, Verticillium, Ustilago, Claviceps, Tilletia, Phoma, Cochliobolus, Gaeumanomyces, Rhychosporium, Biopolaris, and Helminthosporium, and combinations thereof.

33. The use according to any of items 27 to 32, wherein the plant is selected from the group consisting of a crop, a monocotyledonous plant, a dicotyledonous plant, a tree, an herb, a bush, a grass, a vine, a fern, a moss, and green algae.

34. The use according to any of items 27 to 32, wherein the plant is selected from the group consisting of wheat, barley, oats, small cereal grains, oilseed rape, corn, rice, sugar cane, soybean, potato, carrot, coffee and banana.

35. A Kit, comprising the composition, as defined in any one of items 1 to 25, or obtainable from a process as defined in item 26, instructions and a suitable recipient.

36. The kit according to item 35, wherein the kit further comprises one or more active ingredients selected from the group consisting of an insecticide, fungicide, nematicide, bactericide, herbicide, plant extract, plant growth regulator, a plant growth stimulator, and fertilizer.

37. The kit according to item 36, wherein said composition and said one or more active ingredients are provided in separate compartments in the container.

The illustrative examples presented below serve to better describe the present disclosure. However, the formulations described merely refer to some means to some embodiments and should not be taken as limiting the scope of protection.

EXAMPLES

Trials have been conducted to evaluate the effect on precipitation and viscosity of the composition disclosed.

EXAMPLE 1

Preparing suspension concentrates (SC)

In Table 1, an overview of the different ingredients that were used for making suspension concentrates (SC) formulations are provided, together with a description of their functionalities. The composition of four different suspension concentrate (SC) formulations, with or without EDTA are summarized in Table 2. Table 3 contains the SC formulations SC3 with or without EDTA which were used for viscosity measurements (Example 4).

The first step for SC preparation is to dissolve the chelating agent in water. To this treated water the ingredients in Table 1 are added in the following order: PG, Morwet D425, AG6202, Ethylan CO120, and SAG 1572. This mixture is stirred magnetically until everything gets dissolved to form a homogeneous solution. This might take between 10 to 30 mins depending on the volume of the suspension concentrate desired. The vessel should be covered during the mixing process to prevent evaporation of water. When the solution is homogeneous the spore powder is added while the solution is stirred using a high shear mixer (IKA T25 digital Ultra Turrax, T25-D 550002923) at a speed 10 x1000

rpm for 5-30 min. Finally, citric acid solution is added dropwise until the pH reaches 4.5-4.7.

The details of the water used for preparing these SC is given in Table 4 and Table 5 (Example 2). The detailed method for preparing standard water C and E can be found in CIPAC method MT. 18.1.3 and MT. 18.1.5 respectively. The hardness of standard water C is 500 ppm and that of standard water E is 1500 ppm. The tap water used in the experiments has a hardness of 320 ppm hardness.

Table 1: Name of ingredients used in the formulations, their suppliers and functionalities.

Ingredients	**Suppliers**	**Ingredient functionality**
Bacillus megaterium Spores	Chr. Hansen A/S	Active ingredient (AI)
Morwet D425	Nouryon	Anionic dispersant
AG6202	Nouryon	Non-ionic dispersant
Berol 904	Nouryon	Non-ionic dispersant
Ethylan CO120	Nouryon	Polymeric stabilizer
Potassium Chloride (KCl)	Merck	Salt to regulate osmolarity
SAG 1572	Momentive	Antifoam
Citric acid	Sigma	pH regulator and chelating agent
Dissolvine Na (EDTA)	Nouryon	Chelating agent (or builder)
Propylene glycol (PG)	Sigma	Co-solvent
Water	See Table 4 and 5 for types of water used for preparing SC formulations	Solvent/dispersion medium

Table 2: Composition of suspension concentrate (SC) formulations SC1 and SC2 with or without EDTA.

Ingredients	**SC1**	**SC1+EDTA**	**SC2**	**SC2+EDTA**

	(% w/w)	(% w/w)	(% w/w)	(% w/w)
Bacillus megaterium spore	26	26	26	26
Morwet D425	3	3	3	3
AG6202	0.3	0.3	0	0
Berol 904	0	0	0.3	0.3
Ethylan CO120	0.5	0.5	0.5	0.5
PG	10	10	10	10
KCl	1	1	1	1
SAG 1572	0.1	0.1	0.1	0.1
Water	54.4	53.9	54.4	53.9
20 % Citric acid	4.7	4.7	4.7	4.7
Dissolvine Na (EDTA)	0	0.5	0	0.5
Total	100	100	100	100

Table 3: Composition of suspension concentrate (SC) formulations SC3 (without EDTA) and SC3+EDTA.

Ingredients	**SC3 (% w/w)**	**SC3+EDTA (% w/w)**
Bacillus megaterium spore	25	25
Morwet D425	2.5	2.5
AG6202	0.3	0.3
Ethylan CO120	0.5	0.5
PG	10	10
SAG 1572	0.1	0.1
Water	54.5	54.0
25% Citric acid	7.1	7.1

Dissolvine Na (EDTA)	0	0.5
Total	100	100

EXAMPLE 2

Wet-sieve analysis of suspension concentrates (SC) by CIPAC methods

The SC formulations (Table 2) were characterized by CIPAC methods (Collaborative International Pesticides Analytical Council Limited, Volume K, 2003 and volume F, 2007 versions).

Wet-sieve analysis:

In this test a sample of the formulation is dispersed in water and the suspension formed is transferred to a sieve (75 µm mesh size/200 mesh size according to ASTM E 11-61, 0.075 mm according to ISO 565, DIN 4188) and washed. The amount of the material retained on the sieve is determined by drying and weighing (CIPAC method MT 185).

Wet sieve tests (CIPAC MT. 185) were performed on each of the formulation as tabulated in Table 4. 10 g of each formulation was added to standard water C (500 ppm hardness), standard water E (1500 ppm hardness) and tap water (320 ppm hardness) and stirred using a magnetic stirrer for 5 min. Next, each dilution was washed on a 75 µm mesh with standard water C or standard water E or tap water. Wet sieve residue was dried in the vacuum oven at 55 °C until constant weight is achieved.

Percentage (%) of Wet-sieve residues of SC1 formulations with or without EDTA are reported in Table 4 and Table 5. SC1 formulations were prepared in either MQ water or water having 500 ppm hardness. Water of four different hardness was used to dilute the SC formulations, namely, 0, 320, 500 or 1500 ppm. The pH values were in the range 6 – 8 and the ratio Ca^{2+}: Mg^{2+} was 4:1. Finally, the formulations were washed on a 75 µm mesh using tap water of 320 ppm hardness.

From table 4, it is clear to see that the % of wet-sieve residues either became zero or significantly less for formulations containing EDTA as opposed to SC without EDTA. Moreover, there is a correlation between the residues obtained and the hardness of the water used to dilute the SCs. Water with 1500 ppm hardness resulted in 0.85% residues whereas water with 320 ppm hardness yielded 0.46% residues. In the three formulations containing EDTA the wet-sieve residues were reduced to zero. This clearly shows that the chelating agent is binding with the calcium and preventing it from binding

to ingredients such as citric acid and anionic surfactants. Similar results were obtained for formulation SC2, which is collated in Table 5.

Table 4: Residues obtained after wet-sieve tests with SC1 and SC1+EDTA formulations.

SC formulation	Hardness of water used for making SC (ppm)	Hardness of water used for dilution of SC (ppm)	Hardness of water used for wet-sieve test (ppm)	Residue obtained after wet-sieve test (% w/w)
SC1	500	500	320	0.44
SC1	0	500	320	0.55
SC1+EDTA	500	500	320	0.00
SC1	500	320	320	0.27
SC1	0	320	320	0.46
SC1+EDTA	500	320	320	0.00
SC1	500	1500	320	0.30
SC1	0	1500	320	0.85
SC1+EDTA	500	1500	320	0.10

Table 5: Residues obtained after wet-sieve tests with SC2 and SC2+EDTA formulations.

SC formulation	Hardness of water used for making SC (ppm)	Hardness of water used for dilution of SC (ppm)	Hardness of water used for wet-sieve test (ppm)	Residue obtained after wet-sieve test (% w/w)
SC2	500	320	320	0.50
SC2	0	320	320	0.54
SC2+EDTA	500	320	320	0

It should be highlighted that even for formulations which were made in water having 500 ppm hardness (see Table 4 and 5), the residues could be reduced to zero when diluted with 500 ppm or 320 ppm water. The concentration of EDTA was kept fixed at

0.5% even for the formulations with water having a hardness of 500 ppm and still a significant reduction in residue was achieved. This demonstrates that this concentration of chelating agent may be able to solve the problems associated with applications even in regions having high water hardness (between 500 and 1500 ppm).

Pictures of residues obtained after wet-sieve analysis of SC1 and SC1 + EDTA are shown is Figure 1 (a) and (b), respectively. In 1(a) a significantly higher amount of residues can be seen than in 1(b).

EXAMPLE 3

Analysis of wet-sieve residues obtained after CIPAC method

The wet sieve residues obtained after CIPAC test MT-185 were characterized by taking picture of the residues remaining after drying of the petri dishes. The residues obtained from SC formulation without EDTA were further characterized by scanning electron microscopy (SEM) and their atomic composition was found by energy dispersive x-ray spectroscopy (EDX). The exact chemical structure of the residue was obtained by x-ray powder diffraction (XRD). Finally, a dissolution test was performed by suspending 20 mg of dry residue in either MQ-water or in 0.1 M EDTA (Dissolvine Na) solution. After stirring the residues for 5 min on a magnetic stirrer at room temperature, a picture was taken using a digital camera (Figure 1).

The stereo microscope image of the residues can be seen in Figure 2(a). From the images it is observed that the powder consists of round agglomerates of elongated plate-like crystals. We see the same kind of crystals in our water based SCs containing citric acid. Even though MQ water is used for making the SCs, calcium can be present in the bulk from other co-formulants and also in the diluent water thereby forming calcium citrate. Calcium can also come from the bacterial spores themselves.

Scanning electron microscopy (SEM) equipped with X-ray microanalysis facilities (EDX) allow microstructure and elemental composition of the material to be analyzed. Structure and element composition are documented by SEM-images (Figure 2(b)) and X-ray spectra (Figure 2(c)). The EDX-spectrum in figure 2(c) shows that the sample contains carbon (C), calcium (Ca), oxygen (O), and small amounts of chlorine (Cl), sulphur (S), sodium (Na), silicon (Si), potassium (K), copper (Cu), and phosphorus (P).

X-ray powder diffraction was carried out using an X-ray diffractometer (XRD) equipped with a Cu X-ray source (Kα wavelength 1.5406 Å, Figure 2(d)). This instrument allows the crystalline phases in the powder samples to be analyzed (identification and relative

quantification of phases). The results are compared to reference patterns in the PDF-4+ database (2020 version). The analysis is documented as XRD-patterns in Figure 2(e).

The chemical structure of the calcium citrate tetrahydrate is shown in Figure 2(f). Citric acid contains three carboxylic groups in its structure with three different pK_a: 3.13, 4.76 and 6.4 respectively. The pH of the SC is ~ 4.7. This means that in the formulation only one carboxylic group will be ionized. However, after dilution, if the pH is increased around 6 or 7, all three carboxylic groups will be ionized, and this ionization leads to the formation of calcium citrate tetrahydrate.

It was attempted to dissolve calcium citrate using a chelating agent in water. We chose sodium EDTA (Dissolvine) as EDTA is a strong chelating agent. 20 mg calcium citrate was dissolved in 0.1 M Dissolvine solution (Figure 3(h)). Calcium citrate was completely dissolved as shown in Figure 2(h), however, the same amount of calcium citrate remains insoluble in pure MQ water without Dissolvine (Figure 2(g)).

This shows that sodium EDTA can be used as a chelating agent either in SCs or WPs or WDGs in order to protect the formulation against the harmful effect of hard water. Formulations containing chelating agents, such as sodium EDTA will remain potent even when made or diluted in hard tap water irrespective of the geographical locations. By being able to minimize the amount of precipitation an agricultural formulation composition can be made, which will keep in solution even if hard water is used in the formulation composition or as dilution on site by the user. Precipitate in an agricultural formulation composition needs to be avoid as the particles can block the nozzles of the spray equipment.

EXAMPLE 4

Characterization of Viscosity of SC obtained

When working with agricultural formulation compositions intended for spray application the viscosity is very important. To ensure easy spraying an optimal viscosity is highly appreciated by the user. The viscosity of the SC was therefore determined to evaluate if the chelating agent would modify the viscosity.

The viscosity is measured on a Brookfield DV2T RV rotational viscometer using a small sample adapter. The spindle used was SC4-18. A speed ramp was used to generate different shear rates. All measurements were done at temperature 23 ± 0.3 °C.

EDTA was surprisingly found to be a viscosity modifier of a SC (Figure 3). For example, in the case of SC3, a significantly ($P < 0.05$, two-tailed P value, unpaired t-test, GraphPad Prism 9.0.1) higher viscosity was measured with EDTA as compared to the

SC formulation without EDTA. This finding is truly surprising and remarkable and paves the way for using EDTA as a rheology modifier.

Overall conclusion

Use of chelating agents such as EDTA as an additive in the agricultural formulation composition comprising *Bacillus* ensured the efficacy of the formulated product in hard water and avoided any deteriorating effect caused by the precipitates (residues) formed in the hard water. In addition, the chelating agent (EDTA) was surprisingly found to also modify the rheological properties of the SC, e.g. the viscosity of a certain SC was found to be significantly better.

CLAIMS

1. An agricultural formulation composition for use as a biological product for agricultural application comprising:

   a) a microorganism composition or a consortium of microorganisms in culture medium;

   b) at least one dispersant;

   c) at least one chelating agent or monovalent metal salt thereof;

   and

   d) water

   said composition having a precipitation of between 0.1% and 2% of wet-sieve residues when measured using the CIPAC MT185 method and a viscosity of 10 – 500 cP, when measured on a rotational viscometer at room temperature.

2. The composition according to any preceding claims, wherein said microorganism composition or consortium of microorganisms comprises at least one species selected from the group consisting of *Bacillus* spp., such as *Bacillus megaterium*, *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus paralicheniformis*, *Bacillus amyloliquefaciens*, and *Bacillus licheniformis*.

3. The composition according to any preceding claims, wherein said at least one dispersant is selected from the group consisting of: an anionic dispersant and/or a non-ionic dispersant, such as linear alkyl benzene sulphonates, lignosulfonates, sodium dodecylsulphate, naphthalene sulphonate formaldehyde condensates, sodium polynapthalene sulphonate, salts of ethoxylated phosphate esters, condensed phenolsulphonic acid and their salts, ethoxylated nonyl phenols, polyalkoxylated alcohol, and polysorbates.

4. The composition according to any preceding claims, wherein said at least one chelating agent is selected from the group consisting of Ethylenediaminetetraacetic acid (EDTA), Tetrasodium glutamate diacetate (GLDA), methylglycine diacetic acid (MGDA), ethanoldiglycinic acid (EDG), Diethylenetriaminepentaacetic acid (DTPA), Hydroxyethylethylenediaminetriacetic acid (HEDTA) or any monovalent salt thereof.

5. The composition according to any preceding claims, wherein said water has a hardness of 50 – 1500 ppm.

6. The composition according to any preceding claims, wherein said dispersant is present at 0.001 – 25 % (w/w) in the total composition.

7. The composition according to any preceding claims, wherein said chelating agent or monovalent salt thereof is present at 0.025 – 10 % (w/w) in the total composition.

8. The composition according to any preceding claims, wherein said water is present at 30 – 98.874 % (w/w) in the total composition.

9. The composition according to any preceding claims, wherein said composition comprises from 1.0 x 10^6 CFU/gram to 1.0 x 10^9 CFU/gram of formulation.

10. Process for preparing the composition, as defined in any one of claims 1 to 9, comprising mixing, in desired ratios, the mentioned ingredients.

11. Use of the composition, according to any one of claims 1 to 9, or obtainable from a process as defined in claim 10, as a biological product for agricultural applications on a plant culture.

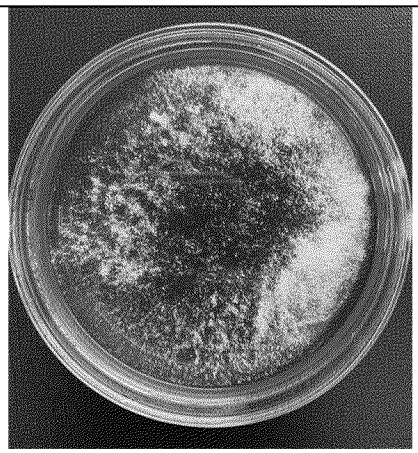
12. Use of the composition according to claim 11, as a biostimulant, bionematicide, biofungicide, or biofertilizer.

13. The use according to claim 11, for preventing, controlling, combating and/or conferring induction of resistance to phytopathogens, such as a plant fungal pathogen or a plant bacterial pathogen.

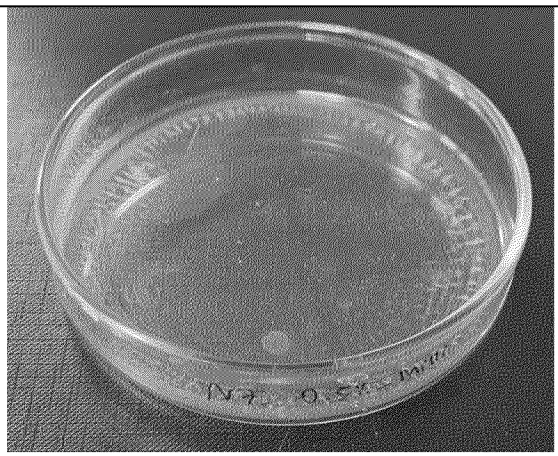
14. A Kit, comprising the composition, as defined in any one of claims 1 to 9, or obtainable from a process as defined in claim 10, instructions and a suitable recipient.

15. The kit according to claim 14, wherein the kit further comprises one or more active ingredients selected from the group consisting of an insecticide, fungicide, nematicide, bactericide, herbicide, plant extract, plant growth regulator, a plant growth stimulator, and fertilizer.

FIGURES



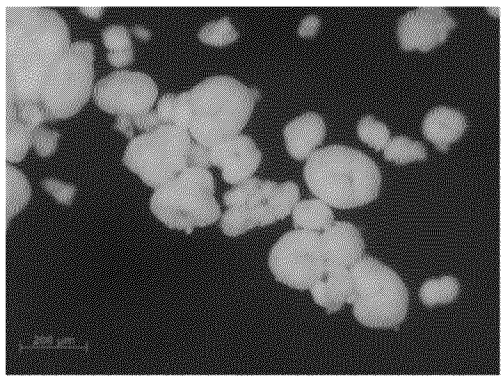
(a)



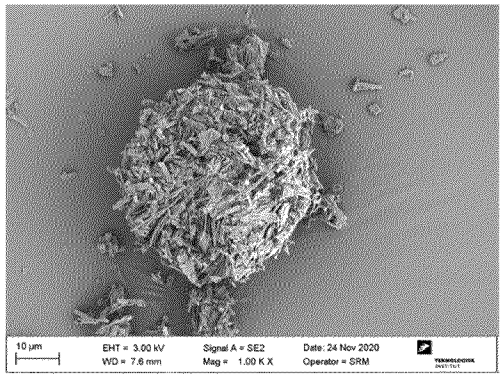
(b)

FIG 1

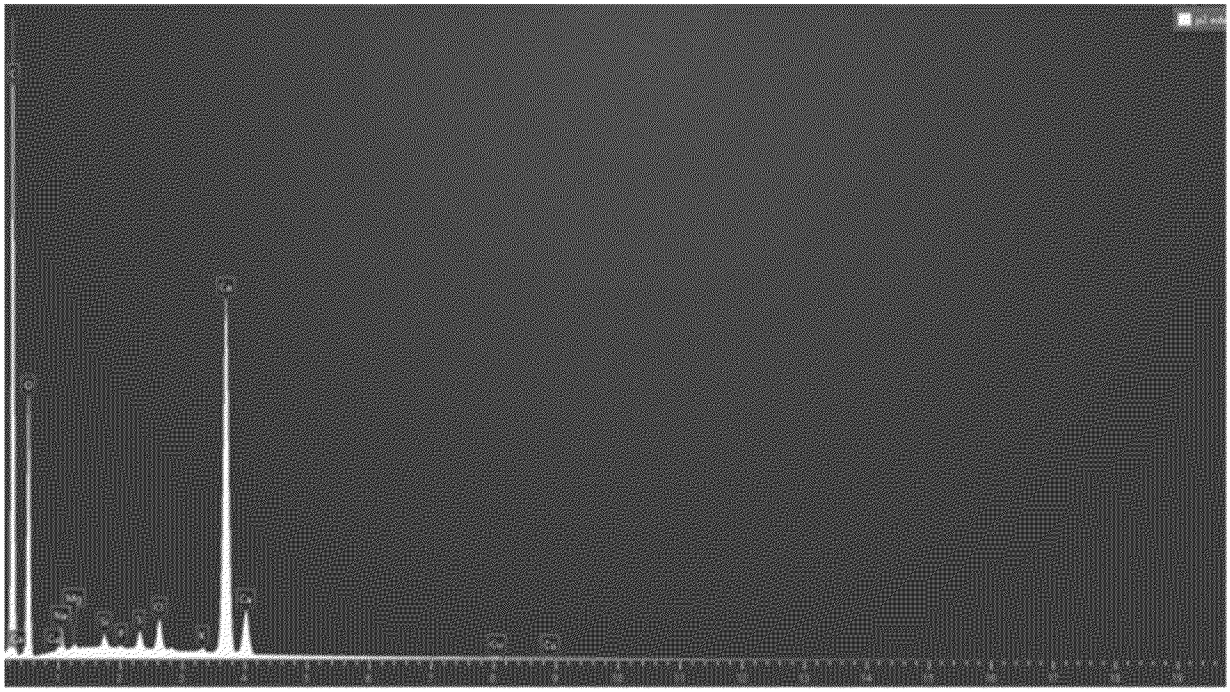
(a)



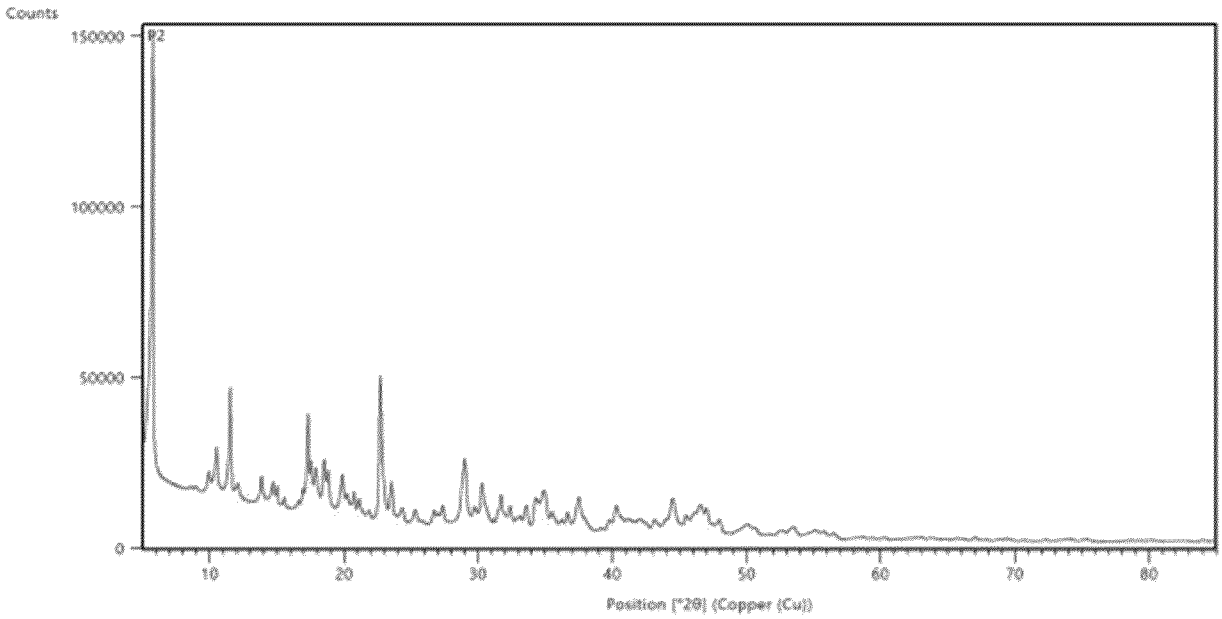
(b)


10 µm
EHT = 3.00 kV
WD = 7.6 mm
Signal A = SE2
Mag = 1.00 K X
Date: 24 Nov 2020
Operator = SRM

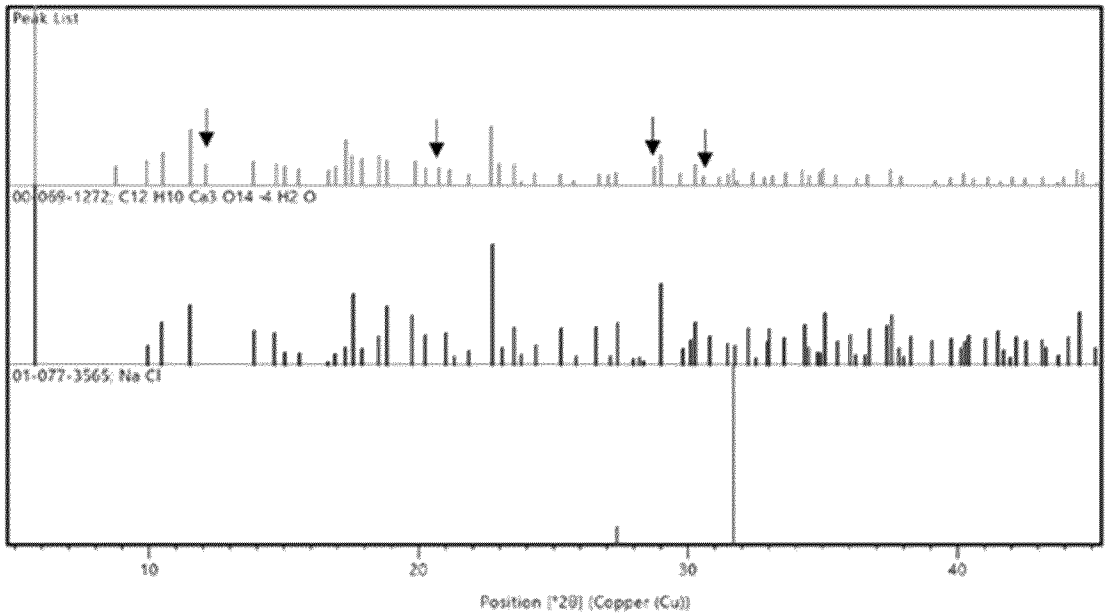
(c)



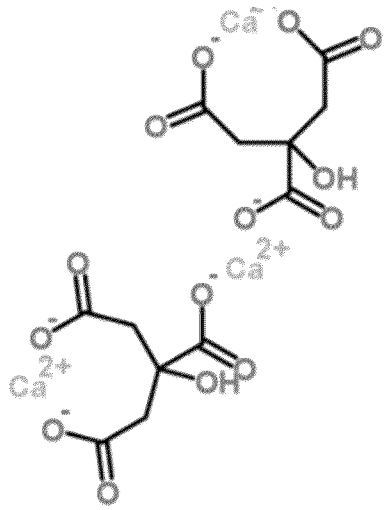
(d)


Counts
150000
100000
50000
0
P2
10
20
30
40
50
60
70
80
Position [°2θ] (Copper (Cu))

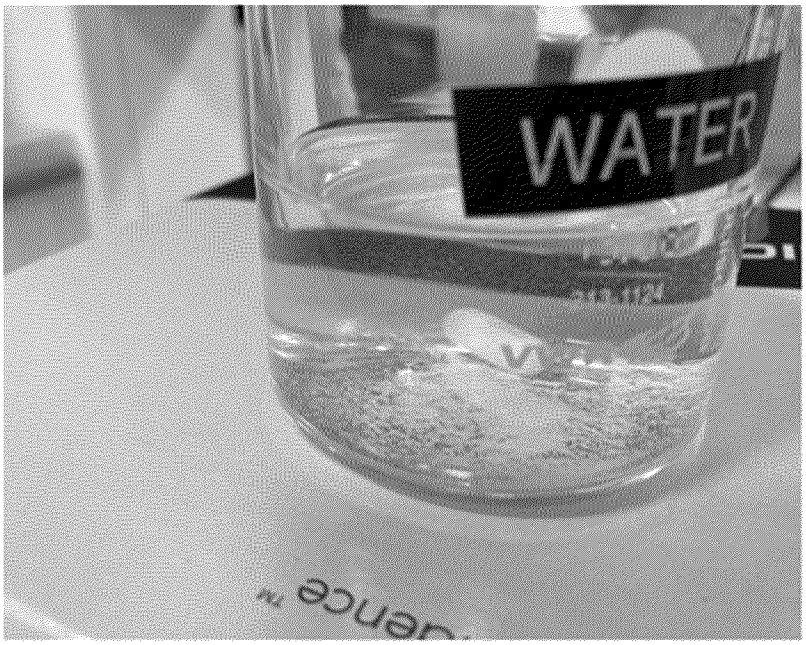
(e)


Peak List
00-069-1272; C12 H10 Ca3 O14 ·4 H2 O
01-077-3565; Na Cl
10
20
30
40
Position [°2θ] (Copper (Cu))

(f)


Ca2+
OH
Ca2+
Ca2+
OH

(g)



(h)

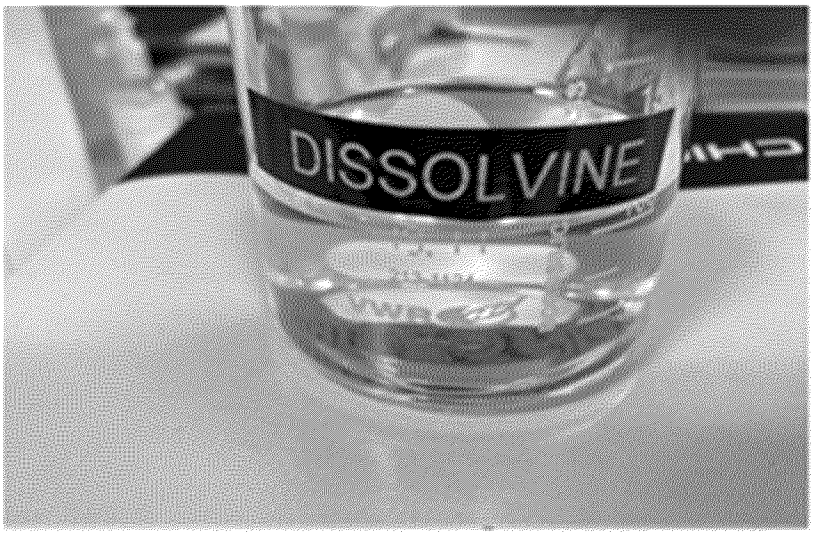


FIG 2

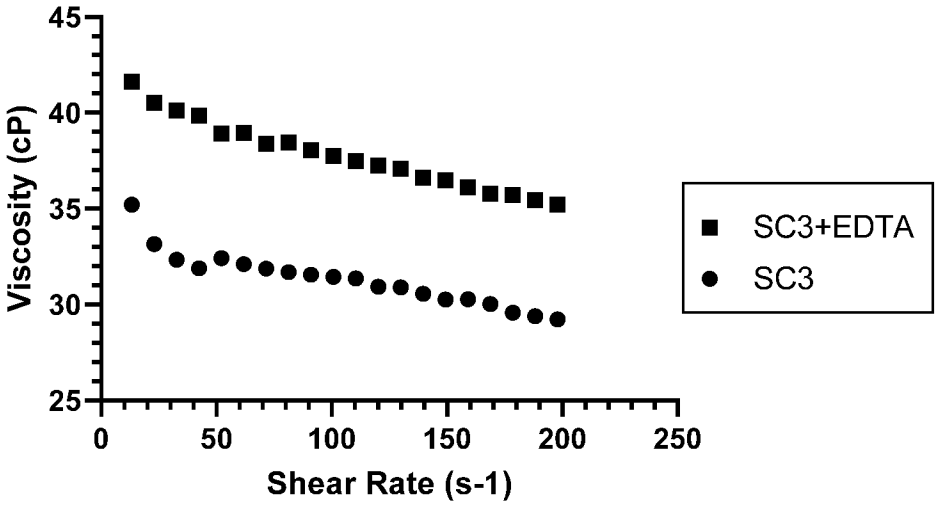

45
40
35
30
25
Viscosity (cP)
0
50
100
150
200
250
Shear Rate (s-1)
SC3+EDTA
SC3

FIG 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/052450

A. CLASSIFICATION OF SUBJECT MATTER
INV. A01N63/22 A01N25/04 A01P3/00 A01P5/00 A01P21/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A01N A01P

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2019/133923 A1 (SUSTAINABLE COMMUNITY DEV LLC [US]) 4 July 2019 (2019-07-04) claims 1-19 paragraphs [0015], [0020] - [0023], [0065], [0077], [0271] - [0296]	1-15
X	WO 2009/126473 A1 (BAYER CROPSCIENCE LP [US]; CHEN CHI-YU ROY [US] ET AL.) 15 October 2009 (2009-10-15) claims 1-18 paragraphs [0032], [0068] - [0071]	1-15
X	WO 2020/247824 A1 (LOVELAND PRODUCTS INC [US]; TENFOLD TECH LLC [US] ET AL.) 10 December 2020 (2020-12-10) claims 1-24	1-15

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 March 2024	17/04/2024

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Marie, Gérald

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/052450

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2022/003113 A1 (LONZA AG [CH]) 6 January 2022 (2022-01-06) cited in the application page 10, lines 21-27 claims 1-13	1-15

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2024/052450

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		US 2023313256 A1	05-10-2023
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