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பொதுசனக் கருத்துரைக்கான கட்டளை வரைவு
DRAFT STANDARD FOR PUBLIC COMMENT

(වෙනස්වීමට ඉඩ ඇත. திருத்தத்திற்குட்படக்கூடியது. Liable to alteration)

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Draft Sri Lanka Standard
SPECIFICATION FOR BIOFERTILIZERS
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இவ்வரைவு இலங்கைக் கட்டளையெனக் கருதப்படவோ அன்றிப் பிரயோகிக்கப்படவோ கூடாது
This draft should not be regarded or used as a Sri Lanka Standard.

අදහස් එවිය යුත්තේ : ශ්‍රී ලංකා ප්‍රමිති ආයතනය, 17, වික්ටෝරියා පෙදෙස, ඇල්විටිගල මාවත, කොළඹ 08.

Comments to be sent to: SRI LANKA STANDARDS INSTITUTION, 17, VICTORIA PLACE, ELVITIGALA MAWATHA, COLOMBO 08.

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මෙම ශ්‍රී ලංකා ප්‍රමිති කෙටුම්පත , ශ්‍රී ලංකා ප්‍රමිති ආයතනය විසින් සකසන ලදුව, සියලුම උද්‍යෝගී අංශ වලට තාක්ෂණික විවේචනය සඳහා යවනු ලැබේ.

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මෙම කෙටුම්පතට අදාල යෝජනා හා විවේචන නියමිත දිනට පෙර ලැබෙන්නට සැලැස්වුවහොත් අගය කොට සලකමු. තවද, මෙම කෙටුම්පත පිළිගත හැකි බැව් හැඟෙන අය ඒ බව දන්වන්නේ නම් එය ආයතනයට උපකාරී වනු ඇත.

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Introduction

This Draft Sri Lanka Standard has been prepared by the Sri Lanka Standards Institution and is now being circulated for technical comments to all interested parties.

All comments received will be considered by the SLSI and the draft if necessary, before submission to the Council of the Institution through the relevant Divisional Committee for final approval.

The Institution would appreciate any views on this draft which should be sent before the specified date. It would also be helpful if those who find the draft generally acceptable could kindly notify us accordingly.

All Communications should be addressed to:

The Director General
Sri Lanka Standards Institution,
17, Victoria Place,
Elvitigala Mawatha,
Colombo 08.

Draft Sri Lanka Standard
SPECIFICATION FOR BIOFERTILIZERS

DSLS:

Gr.

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17, Victoria Place,
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Sri Lanka.

Draft Sri Lanka Standard
SPECIFICATION FOR BIOFERTILIZERS

FOREWORD

This Sri Lanka Standard was approved by the Sectoral Committee on Agriculture and was authorized for adoption and publication as a Sri Lanka Standard by the Council of the Sri Lanka Standards Institution on.....

Biofertilizer is a product containing living soil microorganisms which, when applied to seed, plant surfaces, seedlings or soil, colonizes the endorhizosphere, rhizoplane and / or rhizosphere and /or bulk soil and promotes plant growth by increasing the availability of nutrients and / or providing growth stimulants. Biofertilizers enhance availability of plant nutrients through fixing atmospheric nitrogen, solubilizing of precipitated or mineral forms, and mineralizing of organic forms. They may also stimulate shoot and root growth through the synthesis of growth promoting substances. The use of biofertilizers offer economical and ecological benefits in crop production by way of sustainable use of soil resource and enhancing soil health.

This Standard contributes to ensure the availability of high quality biofertilizers and enables their efficient and effective use. This Standard will also promote the safe use of biofertilizers, promote fair trade and ensure safety of consumers.

This Standard is subjected to the provisions under the Fertilizer Act No. 68 of 1988, the National Environmental Act No. 47 of 1980, the Soil Conservation Act No. 25 of 1951, the Fauna and Flora Protection Ordinance No. 02 of 1937, the Plant Protection Act No. 35 of 1999, the Food Act No. 26 of 1980, the Animal Diseases Act No. 59 of 1992 and Quarantine and Prevention of Diseases Ordinance No. 3 of 1897, and the regulations andamendments framed thereunder, and any other regulatory and statutory requirements wherever applicable.

Guidelines for the determination of compliance of a lot to the requirements of this Standard based on statistical sampling and inspection are given in Appendix **A**.

All values given in this Standard are in SI units.

For the purpose of deciding whether a particular requirement of this Standard is complied with, the final value, observed or calculated, expressing the results of a test shall be rounded off in accordance with **SLS 102**. The number of significant figures to be retained in the rounded off value shall be the same as that of the specified value in this Standard.

In the preparation of this Standard, the valuable assistance derived from the following publications is gratefully acknowledged:

EU 2019/1009	EU Fertilising Products Regulation
GB 20287	Microbial Inoculant in agriculture
IS 8268	*Rhizobium* Inoculants – Specification
IS 9138	*Azotobacter* Inoculants – Specification
IS 14806	*Azospirillum* inoculants
IS 14807	Phosphate Solubilizing Bacterial Inoculant - Specification
IS 15849	Phosphate solubilizing fungal inoculants - *Aspergillus* specification

IS 17134	Liquid Based *Rhizobium* Inoculants – Specification
IS 17135	Liquid Based *Azotobacter* Inoculants – Specification
IS 17136	Liquid Based *Azospirillum* Inoculants – Specification
IS 17137	Liquid Based Phosphate Solubilizing Bacterial Inoculants Specification
IS 17672	Potash Mobilizing Bacterial Inoculant - Specification
IS 17755	Consortia of Microbial Inoculants - Specification
KS 2356	Bio-fertilizer – Specification
PNS BAFS 183	Organic soil amendments

1 SCOPE

1.1 This Standard specifies the requirements and methods of sampling and tests for biofertilizers.

1.2 This Standard does not cover biofertilizers those derived from alien (non-indigenous) and genetically modified microorganisms.

1.3 This Standard is not applicable for liquid fertilizer or liquids that contain solely plant growth regulators or plant growth promoting substances.

2 REFERENCES

SLS	83	SI units and recommendations for use of their multiples and of certain other units
SLS	102	Rules for rounding off numerical values
SLS	428	Random sampling methods
SLS	516	Methods of test for microbiology of food and animal feeding stuffs Part 5: Horizontal method for the detection of *Salmonella* spp. Part 6/ Section 1: Horizontal method for the enumeration of coagulase – positive staphylococci (*Staphylococcus aureus* and other species) - Technique using Baird – Parker agar medium Part 7/ Section 1: Horizontal method for the detection of potentially enteropathogenic *Vibrio* spp. - Detection of *Vibrio parahaemolyticus* and *Vibrio cholerae* Part 12: Horizontal method for the detection and enumeration of presumptive *Escherichia coli* Part 15/Section 1: Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria* spp. – Detection method
SLS	1324	Requirements for organic agriculture production and processing
SLS	645	Methods of test for fertilizers and soil conditioners Part 2: Determination of Moisture content Part 8: Determination of pH
DSLS CEN/TS	17702	Plant biostimulants - Sampling and sample preparation

		Part 1: Sampling
		Part 2: Sample preparation
DSLS CEN/TS	17710	Plant biostimulants - Detection of *Listeria monocytogenes*
DSLS CEN/TS	17711	Plant biostimulants - Detection of *Vibrio* spp.
DSLS CEN/TS	17712	Plant biostimulants - Detection of *Staphylococcus aureus*
DSLS CEN/TS	17716	Plant biostimulants - Determination of *Escherichia coli*
DSLS CEN/TS	17717	Plant biostimulants - Detection of *Salmonella* spp.
DSLS ISO	17318	Fertilizers and soil conditioners - Determination of arsenic, cadmium, chromium, lead and mercury contents

3 DEFINITIONS

For the purpose of this Standard, the following definitions shall apply:

3.1 azospirillum inoculants (AZI): A product having single or multiple strains of *Azospirillum* which shall grow and multiply in rhizosphere of the target non-leguminous plant/sand provide biologically fixed nitrogen to target plant/s and enhance, crop protection, growth and yield.

3.2 azotobacter *i*noculants (AI):A product having single or multiple strains of *Azotobacter* which shall grow and multiply in rhizosphere of a particular non-leguminous plant/ s and provide biologically fixed nitrogen to target plant/ s and enhance growth and yield.

3.3 batch: Biofertilizers that is produced from the same materials, at the same time and location, by the same manufacturer/ producer, or made during the same cycle or period of manufacture.

3.4 biofertilizer: A formulation of a single or a consortium of beneficial and viable indigenous soil microorganisms that enhance growth or other metabolic processes and yield of a target crop by either replacing soil nutrients and/ or by making nutrients more available to plants and/ or by increasing plant access to nutrients. They shall pose no threat to health of humans or animals or to the soil biodiversity and environment.

3.5 biofertilizer containing a consortia of microorganisms: A product having two or more beneficial plant growth promoting microorganisms that enhance growth or other metabolic processes and yield of a target crop/ s.

3.6 carrier: Substances/ media/ compounds that can sustain viable forms of microorganisms.

3.7 consortium: A community of microorganisms comprising interacting populations of two or more species or strains that are isolated from natural environments and whose composition is maintained without manipulation and/ or adulteration.

3.8 indigenous soil microorganisms: Native soil microorganisms inhabiting naturally in soils of Sri Lanka from which biofertilizers are manufactured / formulated and being registered at an applicable regulatory authority.

3.9 leguminous plants: Plants of the family *Fabaceae*, which are nodulated by a host specific strain of symbiotic nitrogen fixing bacteria.

3.10 microbial active agent: Beneficial microorganisms in biofertilizers (bacterium, algae, fungus, protozoan, virus, mycoplasma, and rickettsia but excluding nematodes) and any associated metabolites, which support plant growth, improve soil fertility and soil health.

3.11 microbial inoculants: Commercial scale preparations containing optimum population of one or a combination of active strains of beneficial microorganisms in a viable state, intended for seed, seedling or soil application.

3.12 mineral solubilisation: The dissolution of precipitated and / or mineral forms of nutrients and release of plant available forms of nutrients by microbial activity.

3.13 mycorrhizal inoculants (MI): A product having single or multiple strains of mycorrhizal fungi which can colonize root systems of a target plant and thereby enhances nutrient uptake, growth and yield of the inoculated plants.

3.14 nitrogen fixation: A biological process by which the atmospheric nitrogen gas is converted into more reactive nitrogen compounds by nitrogen fixing bacteria.

3.15 non-leguminous plants: Plants other than the members of family Fabaceae.

3.16 phosphate solubilizing microbial inoculants (PSMI): A product having single or multiple strains of bacteria and/ or fungi intended to solubilize insoluble forms of phosphate in the soil.

3.17 potassium mobilizing bacterial inoculants (KMBI): A product having single or multiple strains of bacteria intended to solubilize/ mobilize insoluble potassium in the soil.

3.18 rhizobium inoculants (RI): A product having a single or multiple strains of symbiotic nitrogen fixing bacteria targeting a specified leguminous plant species or a group of leguminous plants which shall produce active root nodules on target plants and thereby enhance nitrogen fixation, growth and yield.

3.19 root nodules: Outgrowths on roots and stems of leguminous plants resulting from the invasion of symbiotic nitrogen fixing bacteria, which facilitate nitrogen fixation.

4 TYPES

Biofertilizers shall be of the following types:

a) Liquid;
b) Powder; and
c) Granular.

5 REQUIREMENTS

5.1 General requirements

5.1.1 The product shall be manufactured by a process adhering to Good Manufacturing Practices (GMP) requirements as prescribed in Sri Lanka Standards.

5.1.2 The raw materials used in manufacturing biofertilizers shall be in accordance with the substances listed in the Appendix **A** of the **SLS 1324**.

5.1.3 The product shall contain effective strain/ s in minimum recommended population/ s of indigenous soil microorganisms.

5.1.4 The product shall not contain any organism other than the microorganism/ s specified in the product label.

5.1.5 The product shall not contain any organisms or toxic substances which would be harmful or injurious to human, plant, animal, other biota and ecosystems.

5.1.6 Powder products shall be loose and granular products shall be uniform in size.

5.1.7 The carrier material shall be neutralized with calcium carbonate and sterilized shall be in the form of a powder capable of passing through 150 to 212 micron (72 to 100 mesh).

5.1.8 The product shall have at least one of the following beneficial traits;

a) Fix atmospheric nitrogen;
b) Solubilize minerals in soil;
c) Increase the accessibility of nutrients to the targeted plant; and
d) Able to stimulate growth of the targeted plant.

5.2 Strain/species identification requirements

5.2.1 Molecular characterization of the strain/ species of beneficial microorganisms used in the biofertilizer product shall be required.

5.2.2 The producer shall provide the classification and identification report issued by an accredited laboratory or recommended by national authoritative institution.

5.2.3 Self-declaration regarding the safety evaluation reports of the product shall be submitted by the manufacturer/producer when requested by regulators.

5.3 Specific quality requirements for biofertilizers containing single species of nitrogen fixing bacteria

5.3.1 *Biofertilizers containing symbiotic Rhizobium bacteria*

The product shall conform to the requirements given in Table **1**, when tested according to the methods given in Column **4** of the Table **1**.

TABLE 1 – Specific quality requirements for biofertilizers containing *Rhizobium* sp.

Sl No. (1)	**Parameter** (2)	**Requirement** (3)		**Method of test** (4)
		Powder, Granular	Liquid	
i)	pH	6.5-7.5		**DSLS 645 Part 8**
ii)	Moisture content, per cent by mass	30-35	-	**DSLS 645 Part 2**
iii)	Viable cell count of the active agent (at 25-30°C), cfu/ml or cfu/g, min.	5×10^{7}	1×10^{8}	**Appendix B**
iv)	Non-target microorganism contamination, at 10^{-5} dilution	Absent		**Appendix B**
v)	Efficiency character	Shall show >50% in dry mass than control on plant species listed in product label		**Appendix C**
vi)	Nodulation test	Positive		**Appendix C**

5.3.2 *Biofertilizers containing associative symbiotic Azospirillum bacteria*

The product shall conform to the requirements given in Table **2**, when tested according to the methods given in Column **4** of the Table **2**.

TABLE 2 – Specific quality requirements for biofertilizers containing *Azospirillum* spp.

Sl No. (1)	**Parameter** (2)	**Requirement** (3)		**Method of test** (4)
		Powder, Granular	liquid	
i)	pH	6.5-7.5		**DSLS 645 Part 8**
ii)	Moisture content, per cent by mass	30-40	-	**DSLS 645 Part 2**
iii)	Viable cell count of the active agent (at 25-30°C), cfu/ml or cfu/g, min.	5×10^{7}	1×10^{8}	**Appendix F**
iv)	Non-target microorganism contamination, at 10^{-5} dilution	Absent		**Appendix F**
v)	Efficiency character	Formation of white pellicle in semisolid nitrogen free bromothymol blue media		**Appendix M**
vi)	Root growth	Shall show >10% in dry mass than control on plant species listed in product label		**Appendix M**

5.3.3 *Biofertilizers containing free living Azotobacter spp***.**

The product shall conform to the requirements given in Table **3**, when tested according to the methods given in Column **4** of the Table **3**.

TABLE 3 – Specific quality requirements for biofertilizers containing *Azotobacter* spp.

Sl No. (1)	**Parameter** (2)	**Requirement** (3)		**Method of test** (4)
		Powder, Granular	liquid	
i)	pH	6.5-7.5		**DSLS 645 Part 8**
ii)	Moisture content, per cent by mass	30-40	-	**DSLS 645 Part 2**
iii)	Viable cell count of the active agent (at 25-30°C), cfu/ml or cfu/g, min.	5×10^{7}	1×10^{8}	**Appendix G**
iv)	Non-target microorganism contamination, at 10^{-5} dilution	Absent		**Appendix G**
v)	Efficiency character	Shall be capable of fixing at least 10 mg of nitrogen per g of sucrose consumed		**Appendix H**

5.4 Specific quality requirements for biofertilizers containing single species of phosphate solubilizing bacteria or fungi

The product shall conform to the requirements given in Table **4**, when tested according to the methods given in Column **4** of the Table **4**.

TABLE 4 – Specific quality requirements for biofertilizers containing phosphate solubilizing bacteria or fungi

Sl No. (1)	**Parameter** (2)	**Requirement** (3)		**Method of test** (4)
		Powder, Granular	liquid	
i)	pH	6.5-7.5	5.0 – 7.5	**DSLS 645 Part 8**
ii)	Moisture content, per cent by mass	30-40	-	**DSLS 645 Part 2**
iii)	Viable cell or spore count of the active agent (at 25-30°C), cfu/ml or cfu/g, min.	5×10^{7}	1×10^{8}	**Appendix B**
iv)	Non-target microorganism contamination, at 10^{-5} dilution	Absent		**Appendix D**

v)	Efficiency character	Shall have phosphate solubilizing capacity in the range of minimum 30%, when tested spectrophotometrically or minimum 5 mm halo zone in prescribed media having at least 3 mm thickness	**Appendix E**

5.5 Specific quality requirements for biofertilizers containing mycorrhizal inoculants

The product shall conform to the requirements given in Table **5**, when tested according to the methods given in Column **4** of the Table **5**.

TABLE 5 – Specific quality requirements for biofertilizers containing mycorrhizal inoculants

Sl No. (1)	**Parameter** (2)	**Requirement** (3)	**Method of test** (4)
i)	pH	6.5-7.5	**DSLS 645 Part 8**
ii)	Moisture content, per cent by mass	8-12	**DSLS 645 Part 2**
iii)	Total viable spores/g of product, min.	10^2	**Appendix J**
iv)	Infectivity potential, infection points in test roots/g of product, min.	80	**Appendix K**

5.6 Specific quality requirements for biofertilizers containing single species of potassium mobilizing bacteria

The product shall conform to the requirements given in Table **6**, when tested according to the methods given in Column **4** of the Table **6**.

TABLE 6 – Specific quality requirements for biofertilizers containing potassium mobilizing bacteria

Sl No. (1)	**Parameter** (2)	**Requirement** (3)		**Method of test** (4)
		Powder, Granular	liquid	
i)	pH	6.5-7.5	5.0 – 7.5	**DSLS 645 Part 8**
ii)	Moisture content, per cent by mass	30-40	-	**DSLS 645 Part 2**
iii)	Viable cell count of the active agent (at 25-30°C), cfu/ml or cfu/g, min.	5×10^7	1×10^8	**Appendix N**
iv)	Non-target microorganism contamination, at 10^{-5} dilution	Absent		**Appendix N**

v)	Efficiency character	Minimum 10 mm solubilisation zone in prescribed media having at least 3 mm thickness.	**Appendix P**

NOTE: *The appropriate method of test method given in Column (4) of the Table 1-6 shall be selected by the analyst as per the organisms declared by the manufacturer/ producer.*

5.7 Specific quality requirements for biofertilizer containing a consortia of microorganisms

5.7.1 The product shall conform to the requirements given in Table **7**, when tested according to the methods given in Column **4** of the Table **7**.

TABLE 7 – Specific quality requirements for biofertilizer containing a consortia of microorganisms

SI No. (1)	**Parameter** (2)	**Requirement** (3)		**Method of test** (4)
		Powder, Granular	liquid	
i)	pH	5.0-7.5		**DSLS 645 Part 8**
ii)	Moisture content, per cent by mass	30-40	-	**DSLS 645 Part 2**
iii)	Viable cell or spore count (at 25-30°C), cfu/ml or cfu/g, min.	1×10^{6} each of the constituent microorganism individually		**Appendix Q**
iv)	Non-target microorganism contamination, at 10^{-5} dilution	Absent		**Appendix Q**

5.7.2 Individual microorganism present in biofertilizer containing a consortia of microorganisms as per label claim, shall demonstrate minimum bio efficacy value as follows:

a) *Rhizobium*- effective nodulation on all the legume species listed on the label;
b) *Azotobacter*- 10 mg of N fixation/g of sucrose utilized;
c) *Azospirillum* - show >10 % in dry mass than control on plant species listed in product label;
d) Phosphate Solubilizing microbial Inoculant- 10 mm zone of solubilization on PSMI media having at least 3 mm thickness; and
e) Potassium Mobilizing Bacterial Inoculant -10 mm zone of solubilization on KMBI media having at least 3 mm thickness.

The bio efficacy value for *Rhizobium* inoculants shall be tested as described in **Appendix C**; for *Azotobacter* as per **Appendix H** and *Azospirillum* as per **Appendix M**, for PSMI as per **Appendix E**; for KMBI as per **Appendix N** of this Standard.

5.8 Pathogenic limits

The product shall not exceed the pathogenic limit given in Table **8** when tested according to the method prescribed in Column **4** of the Table **8.**

TABLE 8 –Limits for pathogenic organisms for biofertilizers

SI No. (1)	**Test organism** (2)	**Limit** (3)	**Method of test** (4)
i)	*Salmonella*, per 25 g or ml	Absent	**SLS 516: Part 5/ DSLS CEN/TS 17717**
ii)	*Staphylococcus aureus*, per g or ml	Absent	**SLS 516: Part 6/ Section 1/ DSLS CEN/TS 17712**
iii)	*Escherichia coli*, MPN per g or ml	Absent	**SLS 516: Part 12/ DSLS CEN/TS 17716**
iv)	*Vibrio cholerae*, per 25 g or ml	Absent	**SLS 516: Part 7/ Section 1/ DSLS CEN/TS 17711**
v)	*Listeria monocytogenes*, per 25 g or ml	Absent	**SLS 516: Part 15/Section 1/ DSLS CEN/TS 17710**

5.9 Limits for potentially toxic elements

The product shall not exceed the limits for potentially toxic elements given in Table **9**, when tested as prescribed in Column **4** of the Table **9**.

TABLE 9 - Limits for potentially toxic elements for biofertilizers

SI No. (1)	**Elements** (2)	**Limit, mg/kg (maximum)** (3)		**Method of test** (4)
		Liquid	Solid	
i)	Arsenic, as As	0.5	3	**DSLS ISO 17318***
ii)	Cadmium, as Cd	0.5	1.5	
iii)	Chromium, as Cr	0.5	50	
iv)	Lead, as Pb	1	30	
v)	Mercury, as Hg	0.5	0.5	

***NOTE:** *Laboratories may use ICP-MS instead of ICP-OES for detection of potentially toxic elements.*

6 PACKAGING

6.1 The biofertilizers shall be packaged in a well-sealed container which shall not provide deleterious effect on the product from the light, humidity and temperatures.

6.2 All the packaging materials shall be made only of substances, which are safe for living organisms and environment and sufficiently robust to withstand contamination during transportation, handling and storage.

7 STORAGE

7.1 The product shall be stored by the manufacturer in a cool, dry and ventilated place away from direct sun or heat, preferably at a temperature of 15 to 30°C to meet the shelf-life requirement of the product.

7.2 It shall not be stacked in the open air, to prevent the exposure to sun and rain; avoid the influence of adverse conditions.

7.3 It shall also be the responsibility of the manufacturer to instruct the retailers and in turn, the users about the precautions to be taken during storage.

8 MARKING AND/ OR LABELLING

The following shall be marked or labelled legibly and indelibly on each package or container:

a) Name of the product as "BIOFERTILIZERS";
b) Microbial active agent / s shall appear in close proximity to the name of the product by specifying strain / species of the microorganism / s (see **5.2.1**);
c) Count of viable cells / spores;
d) Name and address of the manufacturer / producer;
e) Type / nature of carrier if any;
f) Additives or other substances if any;
g) Registered trade mark if any;
h) Batch or code number;
j) Net content in metric units;
k) Date of manufacture;
m) Date of expiry;
n) Crops for which it is intended;
p) Dilution ratio / application methods and time;
p) Storage instructions; and
q) Safety precautions in handling and application.

9 SAMPLING

Representative samples of the product for ascertaining conformity to the requirements of this Standard shall be drawn as prescribed in Appendix **A**.

10 METHODS OF TEST

10.1 Tests shall be carried out as prescribed in Appendices **B** to **Q** given in this Standard, **Part 5**, **Section 1/ Part 6**, **Section 1/ Part 7**, **Part 12** and **Section1/ Part 15** of **SLS 516**, **Part**

2 and Part 8 of DSLS 645, **DSLS CEN/TS 17717**, **DSLS CEN/TS 17712**, **DSLS CEN/TS 17716**, **DSLS CEN/TS 17711**, **DSLS CEN/TS 17710**, **SLS ISO 17318** and Official Methods of Analysis of the Association of Official Analytical Chemists (**AOAC**).

10.2 Unless otherwise specified, quality reagents, chemicals and distilled water shall be used in tests.

APPENDIX A
COMPLIANCE OF A LOT

The sampling scheme given in Appendix A shall be applied where compliance of a lot to the requirements of this Standard is to be assessed based on statistical sampling and inspection.

Where compliance with this Standard is to be assured based on manufacturer's control systems coupled with type testing and check tests or any other procedure, appropriate schemes of sampling and inspection should be adopted.

A.1 LOT

A.1.1 All units (packages/ containers) in a single consignment of material belonging to the same batch of manufacture or supply shall constitute a lot. If a consignment consists of different batches of the manufacture the containers of the same batch shall be separated and shall constitute a separate lot.

A.1.2 All units in the lot shall consist of similar form (either liquid or solid) of fertilizer packages/ containers.

A.2 GENERAL REQUIREMENTS OF SAMPLING

In drawing, preparing, storing and handling samples, following precautions and directions shall be taken.

A.2.1 Sampling shall be carried out by a trained and experienced person as it is essential that the sample should be representative of the lot to be examined.

A.2.2 The sampling equipment/s shall be perfectly clean, dry and sterile. It shall be properly sterilized by heating in a hot air-oven at 160°C for not less than 2 h or by autoclaving for not less than 20 min at 120°C and held in suitable containers to prevent re-contamination.

A.2.3 Samples in their original unopened containers shall be drawn and sent to the laboratory to prevent possible contamination of sample during handling and to help in revealing the true condition of the material.

A.2.4 The samples shall be protected against extraneous contamination while drawing and handling the samples and to preserve them in their original condition till they are ready for examination in the laboratory.

A.2.5 No preservative or bactericidal/fungicidal agent shall be added to samples required for microbiological analysis

A.2.6 Samples shall be drawn from a protected place not exposed to dampness, air, light, dust or soot.

A.2.7 The sample containers shall be need to be filled only up to 80% of the full volume leaving provision for gas exchange.

A.2.8 The sample containers shall be sealed air-tight after filling and marked with necessary details of sampling.

A.3 SCALE OF SAMPLING

A.3.1 Samples shall be tested from each lot separately for ascertaining conformity of material to the requirements of this Standard.

A.3.2 The sampling shall be drawn as per procedure specified in **DSLS CEN/TS 17702 Part 1**, as appropriate for physical, chemical and biological testing requirements.

A.3.3 The sample preparation shall be done as per the procedure specified in **DSLS CEN/TS 17702 Part 2**, as appropriate for physical, chemical and biological testing requirements.

A.3.4 Unopened package/ container or homogenous sample taken from products in bulk containers shall be selected at random for microbiological testing including strain/species identification.

A.3.5 Preparation of homogenous sample for microbiological testing from products in bulk containers selected as in **A.3.4** shall be conducted as per the procedure given below. Sample shall be drawn from the top, middle and bottom portions of the bulk product using an appropriate sterilized sampling instrument under aseptic condition to form final sample for microbiological tests. The sample shall be put into sterile sample container and marked with necessary details of sampling. The sample shall be approximately 250 ml or 250 g.

A.3.6 The packages or containers shall be selected at random. In order to ensure the randomness of selection, tables of random numbers as given in **SLS 428** shall be used.

A.4 NUMBER OF TESTS

A.4.1 Each package or container shall be selected as in Clause **A.3.2** shall be inspected at the point of sampling for packaging, storage and marking and/or labelling requirements specified in Clause **6**, **7** and **8**.

A.4.2 Each package or container shall be selected as in Clause **A.3.2** and prepared as in Clause **A.3.3** shall be tested for the physical requirement specified in Clause **5.1.6**, requirements of pH and moisture content in Table **1** to **7** (SI no i and ii respectively) of Clause **5.3** to **5.7**, as per the organisms declared by the manufacturer/producer and requirements of potentially toxic element limits specified in Clause **5.9**.

A.4.3 Specimens selected as in Clause **A.3.4** and **A.3.5** shall be tested for the microbiological requirements specified in Clause **5.2.2**, remaining biological requirements specified in Table **1** to **7** (SI no iii onwards) of Clause **5.3** to **5.7**, as per the organisms declared by the manufacture and pathogenic limits specified in Clause **5.8**.

A.5 CRITERIA FOR CONFORMITY

A lot shall be declared as conforming to the requirements of this Standard if the following conditions are satisfied.

A.5.1 Each package or container inspected as in Clause **A.4.1** shall satisfies the relevant requirements.

A.5.2 All test specimens tested as in Clause **A.4.2** shall satisfy the relevant and applicable requirements.

A.5.3 All test specimens tested as in Clause **A.4.3** shall satisfy the relevant and applicable requirements.

APPENDIX B
DETERMINATION OF NUMBER OF *RHIZOBIUM* CELLS

B.1 APPARATUS

B.1.1 *Pipettes*, Graduated, 1 ml, 10 ml.

B.1.2 *Dilution Bottles or Conical Flasks*

B.1.3 *Petri Dishes*, Clear uniform flat-bottomed.

B.1.4 *Hot-Air Oven*, Capable of giving uniform and adequate temperatures equipped with a thermometer, calibrated to read up to 250°C, and with vents suitably located to assure prompt and uniform heating.

B.1.5 *Autoclave*

B.1.6 *Incubator*

B.1.7 *Hand Tally or Mechanical Counting Device*

B.1.8 *pH* Meter

B.2 REAGENTS

B.2.1 *Congo Red*, One percent aqueous solution.

B.2.2 Medium

Use a plating medium of the following composition:

Agar	20 g
Yeast extract	1 g
Mannitol	10 g
Potassium hydrogen phosphate (K_2HPO_4)	0.5 g
Magnesium sulphate ($MgSO_4.7H_2O$)	0.2 g
Sodium chloride (NaCl)	0.1 g
Congo red	2.5 ml
Distilled water	1000 ml
pH	7

(For the preparation of the medium, use ingredients of uniform quality and chemicals of analytical grade; alternatively use an equivalent dehydrated complete medium and follow the manufacturer's instructions.)

B.3 STERILIZING AND PREPARATION PROCEDURE FOR PLATES

B.3.1 Sterilize sampling and plating equipment with dry heat in a hot-air oven, at not less than 160°C for not less than 2 h.

B.3.2 Sterilize the medium by autoclaving at 120°C for 20 min. To permit passage of steam into and from closed containers when autoclaved, keep stoppers slightly loosened, air from within the chamber of the sterilizer should be ejected allowing steam pressure to rise.

B.4 PREPARATION OF PLATING MEDIUM AND POURING

B.4.1 Prepare growth medium in accordance with the composition indicated in **B.2.2**.

B.4.2 Melt the required amount of medium in boiling water or by exposure to flowing steam in partially closed container but avoid prolonged exposure to unnecessarily high temperature during and after melting. Melt enough medium which will be used with in 3h. Re-sterilization of the medium may cause partial precipitation of ingredients.

B.4.3 When holding time is less than 30 min promptly cool the molten medium to about 45°C, and store until used, in a water bath or incubator at 43°C to 45°C. Introduce 12 to15 ml of liquefied medium or appropriate quantity depending on size of the Petri dish at 42 to 44°C into each plate. Gently lift the cover of the dish just enough to pour in the medium. Sterilize the lips of the medium container by exposure to flame:

a) Immediately before pouring;
b) Periodically during pouring; and
c) When pouring is completed for each batch of plates, if portion of molten medium remain in containers and are to be used without subsequent sterilization for pouring additional plates.

B.4.4 By rotating and tilting the dish and without splashing the medium over edge, spread the medium evenly over the bottom of the plate. Provide conditions so that the medium solidifies with reasonable promptness (5-10 min) before removing the plates from surface.

B.5 PREPARATION OF SERIAL DILUTION FOR PLATE COUNT

Dispense 30 g of Inoculants to 270 ml of sterile distilled/ demineralized water and shake for 10 min on a reciprocal shaker or homogenizer. Make serial dilutions up to 10^{-9} by suspending 10 ml aliquot of previous dilution to 90ml of water. Take 0.1 ml or suitable aliquots of 10^{-5} to 10^{-9}dilutions using sterile pipettes and deliver to Petri dishes containing set medium as given in **B.2.2** and spread it uniformly with a spreader. Invert the plates and promptly place them in the incubator.

B.6 INCUBATION OF PLATES

Label the plates and incubate at 28±2 ºC for 3 to 5 days for fast growing Rhizobia and 5 to 10 days for slow- growing ones.

B.7 COLONY COUNTING AIDS

B.7.1 Count the colonies with the aid of magnifying lens under uniform and properly controlled, artificial illumination. Use a colony counter, equipped with a guide plate and rules in Centimeter Square. Record the total number of colonies with the hand tally. Avoid mistaking particles of undissolved medium or precipitated matter, in plates for pinpoint colonies. To distinguish colonies from dirt, specks and other foreign matter, examine doubtful objects carefully.

B.7.2 Count all plates but consider for the purpose of calculation plates showing more than 30 and less than 300 colonies per plate. Disregard colonies, which absorb Congo red and stand out as reddish colonies. *Rhizobium* stands out as white, translucent, glistening and elevated colonies. Count such colony numbers and calculate figures in terms of per gram, of carrier. Also check for freedom from contamination at 10^{-5} dilution.

B.8 IDENTIFICATION

The following culture tests need to be carried out in order to check whether the *Rhizobium* colony as developed in the above incubation is pure or contaminated with common contaminants such as *Agrobacterium*.

a) Growth in alkaline medium: *Agrobacterium radiobacter* can be detected by drawing streaks on Hoffer's alkaline medium (pH 11.0) where *Rhizobium* does not grow, while *A. radiobacter* does.
b) Growth in glucose peptone agar: *Rhizobium* shows little or no growth on glucose.

Gram test: It should test negative.

APPENDIX C
TEST FOR NODULATION

C.1 POT CULTURE TEST

C.1.1 Plant nutrient solution

SI No. (1)	Composition (2)	Conc. (3)	g/l (4)
i)	Potassium chloride	0.001M	0.0745
ii)	Di Potassium hydrogen Phosphate (K_2HPO_4)	0.001M	0.175
iii)	Calcium sulphate ($CaSO_4.2H_2O$)	0.002M	0.344
iv)	Magnesium sulphate ($MgSO_4.7H_2O$)	0.001M	0.246
v)	Trace elements solution		0.5ml
	1. Copper sulphate ($CuSO_4.5H_2O$)	0.01mg/kg	0.78
	2. Zinc Sulphate ($ZnSO_4.7H_2O$)	0.25 mg/kg	2.22
	3. Ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$)	0.0025 mg/kg	0.01
	4. Magnesium sulphate ($MgSO_4.7H_2O$)	0.25 mg/kg	2.03
	5. Boric acid (H_3BO_4)	0.125 mg/kg	1.43
	6. Water		1 lit
	Prepare the solution no (v) consisting of trace elements in one liter of stock solution and add to final nutrient solution at the rate of 0.5 ml per liter.		
vi)	Iron solution		0.5 ml
	1. Ferrous sulphate		5
	2. Citric acid		5
	3. Water		100 ml
	Prepare the solution no. (vi) As 100 ml of stock solution and add final nutrient solution at the rate of 0.5 ml per liter.		

C.2 PREPARATION

Prepare the nutrient solution by weighing out substances (i), (ii) and (iv) and dissolving them in a liter of water. To this solution add 0.5 ml of trace elements solution and 0.5 ml of iron solution. Grind in a mortar 0.344 g of calcium sulphate (iii) to a fine consistency and add to the final nutrient solution. Auto clave the nutrient solution thus prepared, at 120°C for 20 min.

NOTES

1. The nutrient solution may be prepared in the tap water provided the water is soft.

2. The nutrient solution should be shaken well to disperse calcium sulphate before dispensing.

3. If the solution is made up with distilled water, the pH is about 7.2 before autoclaving and falls to 5.5 on autoclaving and rises slowly on standing to about 5.8. However, there is no need to adjust pH. For most tropical legumes; pH of about 6.0 is adequate.

C.3 PROCEDURE

C.3.1 Immerse the seeds in 95 percent alcohol and follow by surface sterilization in freshly prepared chlorine water (for 15 to 20 min) or 0.1 per cent mercuric chloride solution 3 min in a suitable container such as a screw –capped bottle or a test tube with a rubber hung. In case of seeds with tough seed coat, concentrated sulphuric acid may be used as a surface sterilant for 20 to 30 min. It is recommended that the seeds should be placed overnight in a desiccator containing calcium chloride before surface sterilization with sulphuric acid. Pour out the sterilants and wash the seeds in several changes of sterile water and wash the seeds in several changes of sterile water (at least ten times) to get rid of the sterilants. Fill earthenware or glazed pot with soil (2 parts soil and 1 part washed coarse sand) (pH 6 to 7) and autoclave for 2 h at 120^{o}C. After two days' incubation at room temperature, repeat autoclaving to ensure complete sterility of soil. Inoculate surface sterilized seeds with water slurry of the inoculant taken from a culture packet (15 to 100 g seeds per gram of inoculants depending on the size of the seed) and sow the seeds. Keep a set of pots with uninoculated seeds as control and also a set of pots with ammonium nitrate at the rate of 100 kg N/ha as control aid incubate them in a pot-culture house during appropriate seasons for appropriate plants, taking care to separate the inoculated pots from the control pots. If growth rooms or cabinets having facilities to adjust temperature and light are available, the pots may be incubated in such controlled environmental conditions. Sterilize the nutrient solution at 120^{o} C for 20 min and irrigate each pot once to the moisture holding capacity of soil. Subsequently, water the seedling periodically with sterilized water preferably through a plastic tube, taking care to prevent splashing of water from inoculated pots to uninoculated ones. Maintain required number of replicated pots (4 to 16) for each botanical species for statistical analysis.

C.3.2 After two to three weeks of growth, thin down the number of plants in each pot to four uniform plants or as suitable to the pot size. At the end of 6 to 8 weeks, take one set of pots from both the control and inoculated series and, separate the plants carefully from the soil under slow running water. Obtain data on the number, colour (effective nodules are pink or red) and mass of nodules. At the end of 6 to 8 weeks, harvest the shoot system, dry at 60 ^{o}C for 48 h and determine dry mass. For the above purpose, maintain adequate replications of pots (4 to 16).

C.3.3 Record the nodulation data regarding formation of pink colour of nodules as revealed visually when nodules are cut open by razor blade. After computing the data, based on the dry mass of plants and nodulation data decide the effectiveness of culture. If good effective pink nodulation is obtained in inoculated plants together with local absence or sometimes presence of stray nodules in controls and if there is a 50 percent increase in the dry mass of plants over the uninoculated control without nitrate, it may be concluded that the culture is of the require quality.

APPENDIX D
DETERMINATION OF NUMBER OF PHOSPHATE SOLUBILISING MICROBIAL CELLS

D.1 APPARATUS

D.1.1 *Pipettes*, Graduated, 1 ml, 10 ml.

D.1.2 *Dilution Bottles or Conical Flasks*

D.1.3 *Petri Dishes*, Clear uniform flat-bottomed

D.1.4 *Screw-Capped Tubes*

D.1.5 *Incubator*

D.1.6 *Hot-Air Oven*, Capable of giving uniform and adequate temperatures equipped with a thermometer, calibrated to read up to 250 °C, and with vents suitably located to assure prompt and uniform heating

D.1.7 *Autoclave*

D.1.8 *pH Meter*

D.1.9 *Protective Chamber with Burner*

D.1.10 *Haemocystometer*

D.1.11 *Compound Microscope*

D.1.12 *Glass Slides and Cover Slips*

D.1.13 *Forceps*

D.1.14 *Needles*

D.1.15 *Glass Rod*

D.2 REAGENTS

D.2.1 Medium Use a plating medium of the following composition:

Glucose	10.0g
Tri-calcium phosphate	5.0 g
Ammonium sulphate	0.5 g
Magnesium sulphate	0.1 g
Sodium Chloride	0.2 g
Yeast extracts	0.5 g
Manganese sulphate	Trace
Ferrous sulphate	Trace
Distilled water	1000ml
Agar	15.0 g
pH adjusted to	7 ± 0.2

D.3 STERILIZING & PREPARATION PROCEDURE FOR PLATES

The method same as described in **B.3**

D.4 PREPARATION OF PLATING MEDIUM AND POURING

The method same as described in **B.4**

D.5 PREPARATION OF SERIAL DILUTION FOR PLATE COUNTS/ SPORE COUNT METHOD

Dispense 30 g of inoculants in 270 ml of sterile water and shake for 10 min on a reciprocal shaker. Make serial dilutions up to 10^{-7} level. Pipette out 0.2 ml aliquots of 10^{-5} to 10^{-7} dilution and deliver it on the petri dishes containing set medium as described in **D.2.1**. Spread the aliquots over the plate. Invert the plates and place them in the incubator at 28 ± 2 °C for 3 days. Use 3 replicates of 10^{-5}, 10^{-6} and 10^{-7} dilution.

D.6 INCUBATION OF PLATES

Label the plates and incubate at 28±1° C for 4 to 6 days.

D.7 COLONY COUNTING AIDS

The method same as described in **D.7**

D.8 COUNTING

Count the total number of colonies on the plates including colonies with solubilisation zone with the help of a colony counter.

D.9 METHODS OF COUNTING SOLUBILISATION ZONES

D.9.1 Take 10 g of inoculants in 90 ml in water

D.9.2 Make a ten-fold dilution series up to 10^{-7}

D.9.3 Take 0.2 ml aliquote of 10^{-5} to 10^{-7} dilutions using sterile pipettes and delivered to petri dishes containing Pikowskya media (**D.2.1)**.

D.9.4 Spread it uniformly, Invert the plates and incubate them up to 2 weeks at 28 ±2°C

D.9.5 Count the colonies showing hallow cones and measure their diameter.

D.10 IDENTIFICATION

Phosphate-solubilizing micro-organisms can be either bacteria or fungi. The following species are more effective:

Bacteria:

a) *Bacillus megaterium*,
b) *Bacillus polymyxa*,
c) *Bacillus pulvijaciens*,
d) *Pseudomonas striata*,
e) *Pseudomonas rathonis*;

Fungi:
a) *Aspergillus niger*,
b) *Aspergillus awamori*,
c) *Penicillium digitatum*.

The bacterial species are aerobic and heterotrophic. Cell size is 1.1–2.2 μm, and the cells are rod-shaped. The transparent zone around microbial colonies indicate the extent of phosphate solubilization and the effectiveness of the microbes.

APPENDIX E
DETERMINATION OF SOLUBLE PHOSPHORUS USING ASCORBIC ACID METHOD

E.1 PRINCIPLE

Soluble phosphorus forms heteropolymolybdophosphate complex with molybdate ions which on reduction produces a characteristic blue colour measured at 840 to 880 nm.
Considering the higher stability of the ascorbic acid, easiness to handle, higher tolerance to the concentration of interfering ions, possibilities to use it with all types of acids and higher stability of the developed colour (from 10 to 60 min), ascorbic acid instead of stanous chloride is now-a-days used as the reducing agent for the hetropolymolybdophosphate complex formed by the soluble phosphate ions on addition of ammonium molybdate solution.

E.2 APPARATUS

Spectrometer capable of transmission measurement at 840 to 880 mm.
Extractant: It is Olsen extract.

E.3 REAGENTS

E.3.1 *Ammonium Molybdate* $[(NH_4)_6\ Mo_7O_{24}.4H_2O]$

E.3.2 *L- Ascorbic Acid*

E.3.3 *p-Nitro phenol*

E.3.4 NH_2SO_4

E.3.5 *Olsen Extractant*, 0.5 M sodium bicarbonate (NaHCO3) solution at *p*H of 8.5

E.4 PREPARATION OF REAGENTS

E.4.1 Sulphomolybdic Acid

Take 20 g of ammonium molybdate and dissolve in 300 ml of distilled water.
Add slowly 450 ml of 10N H_2SO_4.
Cool the above mixture and add 100 ml of 0.5 percent solution of antimony Potassium tartrate.
Cool and make the volume to one liter.
Store in glass bottle away from direct sunlight.

E.4.2 Preparation of Mixed Reagent

Add 1.5 g of *L*-ascorbic acid in 100ml of the above stock solution and mix.
Add 5ml of this solution to develop colour.
Mixed reagent is to be prepared fresh as it does not keep for more than 24 h.

E.4.3 Procedure

Preparation of Sample- Pure culture medium same as at **D.2.1** above excluding agar. Prepare broth medium in 100 ml aliquots in 6 no., 250 ml conical flasks and sterilize in autoclave at 121°C for 20 min.
Inoculation of Medium - Select one PSB colony of the type that has been counted as PSB (showing sufficient zone of solubilization) and streak on set medium as described at **D.2.1** in a Petri dish. Use this pure culture for inoculating the broth. Inoculate 3 flasks and keep 3 flasks as uninoculated control. Incubate the flasks over rotary shaker for 12 days at 28±1°C.
After 12 days, filter the contents of each flask separately through Whatman No. 42 filter paper or centrifuge at 10,000 rpm for 15 min.
Add 10 ml of filtrate/ centrifugate to 50 ml of Olsen extractant and shake for 30 min over rotary shaker.
Filter the suspension through Whatman filter paper No. 40. If the filtrate is coloured then add a tea spoon of Dacro-60 (activated phosphorous free carbon), reshake and filter.
Take a known aliquot (5 to 25 ml) of the extract in a 50 ml volumetric flask.
Add 5 drops of p-nitrophenol indicator (1.5 per cent solution in water) and adjust the pH of the extract between 2 and 3 with the help of NH_2SO_4. The yellow colour will disappear when the pH of the solution becomes 3. Swirl gently to avoid loss of the solution along with the evolution of CO_2.
When the CO_2 evolution has subsided, wash down the neck of the flask and dilute the solution to about 40 ml.
Add 5 ml of the sulphomolybdic acid mixed reagent containing ascorbic acid, swirl the content and make up the volume.
Measure the transmission after 30 min at 880 nm using red filter. The blue colour developed remains stable up to 60 minutes.
Record the concentration of phosphorous (P) in the extract form the standard curve and calculate the concentration of soluble phosphorous as **E.4.4**.

E.4.4. Calculations

a) Weight of the substance taken = x g
b) Volume of the extract added = 50 ml
c) Volume of the extract taken for P determination = y ml
d) Volume made after colour developed = 50 ml
e) Reading from the standard curve against percent transmission recorded = z ppm
f) Soluble Phosphorous

$$\text{Per cent P} = \frac{Z \times 50 \times 10^{-6} \times 50}{y.x} \times 100$$

E.4.5 Preparation of standard curve

Prepare standard curve using 0.1 to 0.6 ppm P in 50 ml volumetric flask. Plot the standard curve by taking concentration of soluble P on x- axis and percent T on y- axis using a semi log graph paper. It is a straight-line relationship between the soluble P and percent T when plotted on a semilog graph paper.

APPENDIX F
DETERMINATION OF NUMBER OF *AZOSPIRILLUM* CELLS

F.1 APPARATUS

F.1.1 *Pipettes*, Graduated (I ml and 10 ml, capacity)

F.1.2 *Conical flasks*, 150 ml and 250 ml.

F.1.3 *Screw capped tubes of 10 ml capacity*.

F.1.4 *Incubator*

F.1.5 *Petri dishes*

F.1.6 *Hot Air Oven*

F.1.7 *Autoclave*

F.1.8 *pH Meter*

F.1.9 *Visual counting and use of MPN table (MPN table be better kept framed as an apparatus).*

F.2 REAGENTS

Use a medium of the following composition:
NFB Medium (Nitrogen free bromothymol blue medium)

Malic acid	5.0 g
Potassium hydroxide	4.0 g
Di-potassium hydrogen phosphate	0.5 g
Ferrous sulphate	0.05 g
Manganese sulphate	0.01 g
Magnesium sulphate	0.1 g
Sodium chloride	0.2 g
Calcium chloride	0.1 g
Sodium molybdate	0.002 g
Distilled water	1000 ml
Bromothymol blue (0.5% alcoholic solution)	2.0 ml
Agar	1.7 g
pH adjusted to 6.5 - 7.0	

F.3 STERILIZING & PREPARATION PROCEDURE FOR PLATES

The method same as described in **D.3**

F.4 PREPARATION OF SERIAL DILUTION FOR COUNT BY MPN METHOD

Dispense 30 g of ASI in 270 ml of sterile water and shakefor 10minutes on a reciprocal shaker. Make serial dilutions upto 10^{-7} dilution. Pipette out 0.2 ml aliquots of 10^{-5} to 10^{-7} dilution and deliver it to screw cap tubes or petridishes containing set medium as described in **F.2**. Spread the aliquots over the plates. The plates place in the incubator at 28 ± 2°C for 3 days. Use 5 replicates of 10^{-5}, 10^{-6} and 10^{-7} level.

F.5 COUNTING

F.5.1 Count the tubes or plates which have turned blue in colour after inoculation and ascertain the presence of pellicles in undisturbed medium. To determine usual contamination on the same examine doubtful objects carefully.

F.5.2 Count all plates/tubes which have turned blue and consider them for the purpose of calculation. Count such type of tubes/plates and tally this count with MPN table to get the number of cells per gram of the carrier.

$$\textit{Azospirillum} \text{ count /g of carrier} = \frac{\text{MPN table value} \times \text{Dilution level}}{\text{Dry mass of product}}$$

F.6 IDENTIFICATION

Azospirillum organisms are Gram-negative, curved, and rod-shaped of varying size. They contain poly-B-hydroxybutyrate granules. *Azospirillum* shows spiral movements. The formation of white pellicles on semi-solid calcium malate medium is a characteristic of *Azospirillum*. *Azospirillum* forms round-shaped colonies on the solid malate medium. *Azospirillum* micro-organisms are producers of strong bases. Hence, when they grow in a medium containing bromothymol blue indicator, they change the colour of the medium to blue.

Table 10 Most Probable Numbers for use with 10 Fold Dilutions and 5 Tubes per Dilutions (Cochran, 1950)

		Most Probable number for Indicated values of P_2					
P_1	P_2	0	1	2	3	4	5
0	0	0.018	0.036	0.054	0.072	0.090	-
0	1	0.018	0.036	0.055	0.073	0.091	0..11
0	2	0.037	0.055	0.074	0.092	0.11	0..13
0	3	0.056	0.074	0.093	0.11	0.13	0..15
0	4	0.075	0.094	0.11	0.13	0.15	0..17

0	5	0.094	0.11	0.13	0.15	0.17	0..19
1	0	0.020	0.040	0.060	0.080	0.10	0..12
1	1	0.040	0.061	0.081	0.10	0.12	0..14
1	2	0.061	0.082	0.10	0.12	0.16	0..17
1	3	0.089	0.10	0.13	0.16	0.17	0..19
1	4	0.11	0.13	0.15	0.17	0.19	0..22
1	5	0.13	0.15	0.17	0.19	0.22	0..24
2	0	0.046	0.068	0.091	0.12	0.14	0..16
2	1	0.068	0.092	0.12	0.14	0.17	0..19
2	2	0.093	0.12	0.14	0.17	0.19	0..22
2	3	0.12	0.14	0.17	0.20	0.22	0..25
2	4	0.15	0.17	0.20	0.23	0.25	0..28
2	5	0.17	0.20	0.23	0.26	0.29	0..32
3	0	0.078	0.11	0.13	0.16	0.20	0..23
3	1	0.11	0.14	0.17	0.20	0.23	0..27
3	2	0.14	0.17	0.20	0.24	0.27	0..31
3	3	0.17	0.21	0.24	0.28	0.31	0..35
3	4	0.21	0.24	0.28	0.32	0.36	0..40
3	5	0.25	0.29	0.32	0.37	0.41	0..45
4	0	0.13	0.17	0.21	0.25	0.30	0..36
4	1	0.17	0.21	0.26	0.31	0.36	0..42
4	2	0.22	0.26	0.32	0.38	0.44	0..50
4	3	0.27	0.33	0.39	0.45	0.52	0..59
4	4	0.34	0.40	0.47	0.54	0.62	0..69
4	5	0.41	0.48	0.56	0.64	0.72	0..81

5	0	0.23	0.31	0.43	0.58	0.76	0..95
5	1	0.33	0.46	0.64	0.84	1.1	1..3
5	2	0.49	0.70	0.95	1.2	1.5	1..8
5	3	0.79	1.1	1.4	1.8	2.1	2..5
5	4	1.3	1.7	2.2	2.8	3.5	4..3
5	5	2.4	3.5	5.4	9.2	16	-

F.7 CALCULATION

a) To calculate the most probable number of organisms in the original sample, select as P1the number of positive tubes in the least concentrated dilution in which all tubes are positive or in which the greatest number of tubes is +ve, and let P2 and P3 represent the numbers of positivetubesinthe nexttwohigherdilution.

b) Then find the row of numbers in MPN table in which P1 and P2 correspond to the values observed experimentally. Follow that row of numbers across the table to thecolumn headed by the observed value of P.

c) The figure at the point of intersection is the most probable number of organisms in the quantity of original sample represented in the inoculum added in the second dilution. Multiply this figure by the appropriate dilution factor to obtain the MPN value.

APPENDIX G
DETERMINATION OF NUMBER OF *AZOTOBACTER* CELLS

G.1 APPARATUS

Same as described in **D.1**

G.2 REAGENTS

Medium: Use a plating medium of the following composition

Agar	20g
Sucrose ($C_{12}H_{22}O_{11}$)	20g
Ferric sulphate $Fe_2(SO_4)_3$	0.1g
Dibasic potassium phosphate (K_2HPO_4)	1.0g
Magnesium sulphate ($MgSO_4.7H_2O$)	0.5g
Sodium Chloride (NaCl)	0.5g
Calcium carbonate ($CaCO_3$)	2.0g
Sodium Molybdate (Na_2MoO_4)	0.005g
Distilled water	1000ml
pH	6.8 to 7.2

G.3 STERILIZING & PREPARATION PROCEDURE FOR PLATES

The method same as described in **D.3**

G.4 PREPARATION OF PLATING MEDIUM AND POURING

Prepare growth medium in accordance with the composition indicated in **G.2.**

Melt the required amount of medium in boiling water or by exposure to flowing steam in a partially closed container but avoid prolonged exposure to unnecessarily high temperature during and after melting. Do not melt more medium than that will be used within 3 h. Re-sterilization of the medium may cause partial precipitation of ingredients.

When boiling time is less than 30 min, promptly cool the molten medium to about 45°C, and store, until used, in a water bath or incubator at 46 ± 1 °C. Introduce 15 to 20 ml of liquefied medium at 44°C to 45°C into each plate. Gently lift the cover of the dish, only enough to insert the pipette or to pour in the medium. Sterilize the lips of the medium containers by exposure to flame:

a) Immediately before pouring;
b) Periodically during pouring operations;
c) When pouring is complete for each batch of plates; and
d) If portions of molten medium remain in containers and are to be used without subsequent sterilization for pouring additional plate.

As each plate is poured, thoroughly mix the medium in the container.

By rotating and tilting the dish and without splashing the mixture over edge, spread the mixture evenly over the bottom of the plate. Provide conditions so that the mixture solidifies with reasonable promptness before removing the plates from level surface

G.4.1 Preparation of serial dilution for plate counts

Dispense 30 g of Inoculants to 270 ml of sterile distilled/ demineralized water and shake for 10 min on a reciprocal shaker or homogenizer.

Make serial dilutions up to 10^{-9} by suspending 10 ml aliquot of previous dilution to 90 ml of water.

Take 0.1 ml or suitable aliquots of 10^{-5} to 10^{-9} dilutions using sterile pipettes and deliver to Petri dishes containing set medium as given in **G.2** and spread it uniformly with a spreader. Invert the plates and promptly place them in the incubator.

G.5 INCUBATION OF PLATES

Label the plates and incubate at 28 ± 1°C for 4 to 6 days.

G.6 COLONY COUNTING AIDS

G.6.1 Count the colonies, with the aid of magnifying lens under uniform and properly controlled artificial illumination. Use the colony counter equipped with a grid plate rules in centimeter square. Record the total number of colonies with the hand tally. Avoid mistaking particles of undissolved medium or precipitated matter in plates for pinpoint colonies. To distinguish colonies from dirt, specks and other foreign matter, examine doubtful objects carefully.

G.6.2 *Azotobacter* colonies are gummy, raised with or without striations, viscous and often sticky. The pigmentation varies from very light brown to black. Count the colony number and observe the cyst formation as given below and calculate number per gram of the carrier material.

G.6.3 Grow the vegetative cells at 30°C on Burk's agar medium comprising sucrose 20 g; dipotassium hydrogen phosphate 0.64 g; dihydrogen potassium phosphate 0.20 g; sodium chloride 0.20 g; calcium sulphate 0.05 g; sodium molybdate 0.001 g; ferrous sulphate 0.003 g; agar 20 g and distilled water 1 000 ml. Look for vegetative cells after 18 to 24 h either by simple staining method or through a phase contrast microscope.

G.6.4 Grow the cyst cells on Burk's agar medium as given above with 0.2 percent n-butanol in place of the carbon source. Look for cyst formation after 4 to 5 days incubation.

G.7 IDENTIFICATION

Azotobacter has the ability to produce pigment. Different species of Azotobacter produce different pigments, thus facilitating their identification. The pigments produced by important species are:
Azotobacter chroococcum – brown;
Azotobacter beijerinckii – light brown;
Azotobacter vinelandi – greenish yellow;
Azotobacter insignis – light brown;
Azotobacter agilis – green; and
Azotobacter macrocytogenes – purple.

Although pigment production does not give a confirmation of the species, it is a good indication of their presence. The secretion of gum or polysaccharide is another important characteristic, as is the formation of cysts by all the species (for withstanding adverse conditions).

G.8 STAINING AND OBSERVATION UNDER SIMPLE MICROSCOPE

G.8.1 Apparatus

G.8.1.1 *Staining tray*;

G.8.1.2 *Staining rack*;

G.8.1.3 *Slide holder*; and

G.8.1.4 *Disposable gloves*.

G.9 REAGENTS

G.9.1 *Crystal violet 1 percent solution* (primary stain); and

G.9.2 *Copper sulfate 20 per cent* (decolorizer agent).

G.10 PROCEDURE

G.10.1 Prepare a smear from a 12 to 18 h culture with serum protein (to provide a proteinaceous background for contrast).

G.10.2 Allow the smear to air dry. Do not heat fix (to avoid destroying or distorting the capsule or causing shrinkage).

G.10.3 Cover the slide with 1percent crystal violet for 2 min.

G.10.4 Rinse gently with a 20 percent solution of copper sulfate.

G.10.5 Air dry the slide. Do not blot. (Blotting will remove the un-heat-fixed bacteria from the slide and/or cause disruption of the capsule.)

G.10.6 Examine the slide under an oil immersion lens. Bacterial cells and the proteinaceous background will appear purplish while the capsules will appear transparent.

Cysts appeared round, having a discernible outer wall, a much thicker faint blue inner wall (intine) with a circular central body that stained deep purple. Vegetative cells stained uniformly deep purple.

G.11 OBSERVATION UNDER PHASE CONTRAST MICROSCOPE

Under the phase contrast microscope, the center of the cyst appears to be dense and often contains refractile granules. The cyst wall appears as a discrete, non-refractive halo encompassing the body of the cyst. Vegetative cells appear uniformly dense, many with refractive granules.

APPENDIX H
TEST FOR NITROGEN FIXATION IN PURE CULTURE

H.1 PURE CULTURE MEDIUM

Prepare medium as given in **G.2** excluding agar.

H.2 PROCEDURE

H.2.1 Select one *Azotobacter* colony, of the type that has been counted as *Azotobacter* in **G.7,** grow in slant culture on the medium in **G.2.** Use this pure culture for inoculating the broth for nitrogen fixation. For this purpose, take 50 ml aliquots of broth in 250 ml conical flasks for inoculation. Inoculate and incubate for 7 days on a rotary shaker at 29 ± 1°C. Test the contents of the flasks for purity by streaking on fresh medium, Concentrate the contents over a water- bath (50° to 60°C) or in an oven at 90°C or under vacuum at 60°C to dryness. Collect the dried culture and take it as a sample. The contents of the flasks in inoculated control series (flasks inoculated and kept in deep freeze to inhibit growth) should be prepared in a similar manner would serve as control.

H.2.2 Determination by Kjeldahl Method

H.2.2.1 *Reagents*

a) *Sulphuric acid,* 93 to 98 percent N-free.
b) Mercuric oxide, N-free.
c) Potassium sulphate {or anhydrous sodium sulphate)
d) *Sulphide or thiosulphate solution* — Dissolve 40 g commercial potassium sulphide in 1 litre of water (or solution of 40 g sodium sulphide or 80 g sodium thiosulphate in 1 litre may be used).
e) *Zinc granules*
f) *Sodium hydroxide solution N, free* — Dissolve about 450 g solid sodium hydroxide in water, cool and dilute to 1 litre (specific gravity of the solution should be at least 1.36).
g) *Hydrochloric or sulphuric acid standard solution* — Standard solution 0.5 N or 0.1 N in case of amount of nitrogen is small. .
h) Indicators
   i) *Methyl red indicator* — Dissolve 1 g of methyl red in 200 mi of ethanol.
   ii) *Mixed indicator* — Prepare mixed indicator by dissolving 0.8 g of methyl red and 0.2 g of methylene blue in 500 ml of ethanol.
j) *Boric acid,* 4 percent solution,

NOTE

Ratio of salt to acid O/v) should be about i: 1 at the end of the digestion for proper temperature control. Digestion may be incomplete at a lower ratio, and nitrogen may be lost at higher ratio. Each gram of fat consumes 10 ml of sulphuric acid and each gram of carbohydrate I ml of sulphuric acid during digestion.

H.2.2.2 ***Apparatus***

H.2.2.2.1 *For digestion* — Use Kjeldahl's flasks of hard, moderately thick, well-annealed glass with total capacity approximately 500 to 800 ml. Conduct digestion over heating device adjusted to bring 250 ml of water at 25°C to rolling boil in about 5 min. To test the heaters, preheat for 10 min in the case of gas burners, and for 30 min in the case of electric heaters. Add 3 to 4 boiling chips to prevent superheating.

H.2.2.2.2 *For distillation* — Use 500 to 800 ml Kjeldahl's flask fitted with rubber stopper through which passes the lower end of an efficient scrubber bulb or trap to prevent mechanical carry over of sodium hydroxide during distillation. Connect the upper end of the bulb tube to a condenser by rubber tubing. Trap the outlet of the condenser in such a way as to ensure absorption of ammonia distilled over with the receiver.

H.2.2.3 ***Procedure***

H.2.2.3.1 Place 0.25 g of the sample in the digestion flask. Add 0.7 g mercuric oxide, 15 g powdered potassium sulphate or anhydrous sodium sulphate and 25 ml of sulphuric acid. Place flask in inclined position and heat gently until frothing ceases (if necessary add small amount of paraffin to reduce frothing); boil briskly until solution becomes clear and then for 2 h. Cool, add about 200 ml of water, cool to room temperature, add 25 ml of the sulphide or thiosulphate solution and mix to precipitate mercury. Add few zinc granules to prevent bumping.

H.2.2.3.2 Tilt the flask and add 85 ml of sodium hydroxide solution without agitation. Immediately connect the flask to distillation bulb on condenser and with tip of the condenser immersed in 50 ml boric acid solution containing 5-7 drops of indicator, rotate the receiver

flask or mix contents thoroughly. Heat until all ammonia has distilled (collect about 150 ml distillate). Remove receiver, wash tip of condenser and titrate the distillate with standard acid (0-2.2.1.7). Correct for blank determination on reagents.

NOTE

Check the ammonia recording periodically using inorganic nitrogen control, for example, ammonium sulphate.

H.2.2.4 Calculation

$$\text{Nitrogen content, percent by mass, g} = \frac{\text{T V} \times \text{N} \times 1.4}{m}$$

where

TV. - titration value (ml of standard acid);
N = normality of standard acid; and
m = mass of sample taken.

H.2.3 Determination of Sucrose Consumed

H.2.3.1 ***Sample Preparation***

Take 1 g of accurately weighed prepared sample of AI (**H.2.1**) into 250-ml volumetric flask and dilute with about 150 ml of water. Mix thoroughly the contents of the flask and make the volume to 250 ml with water. Centrifuge the mixture for 15 min at 10 000 rpm. Decant the supernatant carefully and estimate the total reducing sugar after inversion **(H.2.3.2)**.

H.2.3.2 ***Method of Inversion***

To 100 ml of the supernatant (**H.2.3.1**) add 1 ml of concentrated hydrochloric acid and heat the solution to near boiling. Keep aside overnight. Neutralize this solution with sodium carbonate and determine the total reducing sugars.

H.2.3.3 ***Determination of Reducing Sugars***

Using separate pipettes, take accurately 5 ml each of Solution A **(H.2.3.4.1)** and Solution B (**H.2.3.4.2**) in porcelain dish. Add about 12 ml of AI solution from a burette and heat to boiling over an asbestos gauze. Add 1 ml of methylene blue indicator and while keeping the solution boiling complete the titration within 3 min, the end point being indicated by change of colour from blue to red. Record the volume (*H)* in ml of AI solution required for titration. Follow the same steps for control. The difference between the amount of total reducing sugars in the control and inoculated expressed in terms of sucrose gives the actual amount of sucrose consumed by the organism. Calculate the amount of nitrogen fixed per gram of sucrose consumed.

H.2.3.4 ***Reagents***

H.2.3.4.1 *Copper sulphate solution (Solution A)* — Dissolve 34.639 g of copper sulphate crystals ($CuSO_4,5H_3O$) in water, dilute to 500 ml and filter through glass wool or filter paper. Standardize the solution as per **H.2.3.4.6**.

H.2.3.4.2 *Potassium sodium tartrate (Rochelle salt) solution (Solution B)* — Dissolve 173 g of potassium sodium tartrate and 50 g of sodium hydroxide in water, and dilute to 500 ml. Let the solution stand for a day, and filter.

H.2.3.4.3 *Hydrochloric acid*, specific gravity 1.18 at 20°C (approximately 12 N).

H.2.3.4.4 *Standard invert sugar solution tm* Weigh accurately 0.95 g of sucrose and dissolve it in 500 ml of water. Add 32 ml of concentrated hydrochloric acid, boil gently for 30 min and keep aside for 24 h. Neutralize with sodium carbonate and make the final volume to 1 000 ml; 50 ml of this solution contains 0.05 g of invert sugar.

H.2.3.4.5 *Methylene blue indicator*, 0.2 percent in water.

H.2.3.4.6 *Standardization of copper sulphate solution* — Using separate pipettes, pipette accurately 5 mi of Solution A **(H.2.3.4.1)** and 5 ml of Solution B **(H.2.3.4.2)** into a conical flask of 250-ml capacity. Heat this mixture to boiling on an asbestos gauze and add standard invert sugar solution **(H.2.3.4.4)** from a burette, about 1 ml less than the expected volume which will reduce the Fehling solution completely (about 48 ml). Add 1 ml of methylene blue indicator while keeping the solution boiling. Complete the titration within 3 min. The end point being indicated by change of colour from blue to red. From the volume of invert sugar solution used, calculate the strength(s) of the copper sulphate solution by multiplying the litre value by 0.001 (g/mi of the standard invert sugar solution).
This would give the quantity of invert sugar required to reduce the copper in 5 ml copper sulphate solution.

H.2.3.5 ***Calculation***

Total reducing sugars, Percent by mass= $\frac{250X100xS}{HXM}$

Where,
S = strength of copper sulphate solution (gof invert sugar),
H = volume in ml of AI solution required for titration, and

APPENDIX J
DETERMINATION OF VIABLE SPORES

J.1 For estimation of viable spores, spores are harvested from the Mycorhizal Inoculum (finished product), stained and observed under stereomicroscope.

J.1.2 Harvesting of Spores from Finished Product Two methods have been specified for harvesting spores – by sieving (**J.1.2.1**) and by sucrose gradient (**J.1.2.2**). The sucrose gradient method shall be taken as the referee method.

J.1.2.1 By sieving

J.1.2.1.1 Apparatus

a) Stalking sieves with nylon or stainless-steel mesh and a large range of pore sizes for isolating spores from the carrier or soil sample
b) 40–50 micron (0.04 mm) sieve for small sized spores
c) 100-micron (0.10 mm) sieve for medium sized spores
d) 250 micron (0.25 mm) sieve for very large spores and sporocarps
e) Wash bottles
f) Jars for collecting the sieving
g) Stereo zoom (stereomicroscope)
h) Petri dishes (11 cm) for observing the sieving under stereomicroscope
j) Micropipettes for spore picking
k) Centrifuge

J.1.2.1.2 Procedure

Mix 5-10 g inoculum in a substantial volume of water and decant through a series of sieves arranged in descending order of mesh size. Roots and coarse debris are collected on a coarse sieve, while spores are captured on one or more finer sieves. Vigorous washing with water is necessary to free spores from aggregates of clay or organic materials. Collect the sieving in jars. Transfer the sieving onto the gridded petri dishes/plate and observe under stereomicroscope after staining as given in **J.1.3.**

J.1.2.2 By sucrose gradient

J.1.2.2.1 Apparatus

a) 40-50 micron (0.04 mm) sieve for small sized spores

b) Wash bottles containing water

c) Jars for collecting the sieving

d) Stereo zoom (stereomicroscope)

e) Petri dishes (11 cm) for observing the sieving under stereomicroscope

f) Micropipettes for spore picking

g) Centrifuge

J.1.2.2.2 Reagents

60 per cent sucrose solution

J.1.2.2.3 Procedure

Collect the sieving by the method at **J.1.1**. Transfer the sieving into centrifuge tubes and centrifuge for 5 minutes at 1750 rpm in a horizontal rotor. Decant the supernatant liquid carefully and re-suspend pellet in 60 percent sucrose solution. Again, centrifuge for 2 –5 minutes. Pour the supernatant (with spores) onto a 300 BSS sieve size and rinse with water to

remove the sugar. Transfer the sieving onto the gridded petri dishes/plate and observe under stereomicroscope after staining as given in **J.1.3**.

J.1.3 Spore staining

J.1.3.1 *Apparatus*

Centrifuge tubes of 1.5-2.0 ml size
Stereomicroscope
Petri-dishes

J.1.3.2 Reagent

3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT)
Distilled water

J.1.3.3 Procedure

Prepare 0.25% solution of 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT). Avoid exposure of MTT solution to light, as the stain is light sensitive. Add freshly collected spores (approximately 100 in number) collected by any of the two methods described at **J.1.2.1** and **J.1.2.2**, to the staining solution and incubate at 27°C in sterile centrifuge tubes in dark. Observe the spores for different colour reactions using stereomicroscope under dark field after 24 hours, 48 hours and 72 hours of incubation. Spores, which stained red or pink, are treated as viable. Count the number of spores in plate/dish and express it as spores/g of the sample

APPENDIX K
ASSESSMENT OF INFECTIVITY POTENTIAL

K.1 PRINCIPLE

The bioassay is used to determine the number of infective propagules present in the product. Once the infective propagules (spores, mycelia and vesicles in the root fragments) meet the host roots they give out a turgid mycelial structure - the appressorium, which is the initial step in the penetration event. This appressorium facilitates the fungus to enter the root through an 'entry point'. This entry point can be visualized by staining and enumerated as a measure of the infectivity of the inoculum. Host plants are grown from pre germinated seeds, and a known weight of the inoculum is applied to the test host plant in pots. These pots are maintained for 15 days if the ambient temperature is >25°C and for 30 days if the ambient temperature is <25ºC, after which they are harvested, the root length measured and then stained. The resulting entry points are counted to evaluate the infectivity potential.

K.2 APPARATUS

K.2.1 *Pots (5 x 7 cm in size)*

K.2.2 *Sorghum seeds (Sorghum vulgare)*

K.2.3 *Scissors and needles*

K.2.4 *Petri dish (gridded)*

K.2.5 *Water bath*

K.2.6 *Glass slides and cover slips*

K.2.7 *Compound microscope*

K.2.8 *Coarse sieve to prevent root loss during washing/changing solutions*

K.2.9 *Plastic vials with tight-sealing lids for storage of stained samples in 50% glycerol*

K.3 REAGENTS

K.3.1 Potassium hydroxide solution (5-10%)

K.3.2 Alkaline H2O2 (3 ml of 25% Ammonia solution + 30 ml of 10% H_2O_2 + 67 ml of distilled water)

K.3.4 1% HCl

K.3.5 50% glycerol-water (v/v) solution for de-staining and storage of stained roots

K.3.6 Lactoglycerol (876 ml of lactic acid + 64 ml of glycerine + 60 ml of distilled water:)

K.3.7 Staining solutions:

a) 0.01 % acid fuschin: 0.01 g acid fuschin in 100 ml lactoglycerol; or
b) 0.05% trypan blue: 0.05 g trypan blue in 100ml lactoglycerol 0.03% ; or
c) Chlorozol black E (CBE) in lactoglycerol (1:1:1 lactic acid, glycerol and water). Dissolve CBE in water before adding equal volumes of lactic acid and glycerol.

K.4 PROCEDURE FOR ASSESSMENT OF INFECTIVITY POTENTIAL

Place 100 g test sample in a pot. Dilute the inoculum with sterilized sand if the inoculum is very rich. Plant 10-12 pre-germinated seeds of sorghum or maize and grow for 30 days if the ambient temperature is >25°C and for 45 days if the ambient temperature is <25°C. Harvest the plants from pots and recover roots (fine roots can be rescued using sieve) completely. Chop the roots equally at 1 cm in length. Measure and record the root length from each sample/dilution as described in **K.4.1**. Clear the roots in KOH solution and stain the root pieces as described in **K.4.2**. Count the number of infection points/entry points formed on randomly picked 100 segments. Calculate the average number of entry points formed in 1 cm segment. Calculate the total number of infection points/infective propagules (IP) by multiplying the average number of entry points formed in 1 cm segment by the total root length. Extrapolate the IP present as numbers per gram of substrate/inoculum

K.4.1 Estimation of root length

K.4.1.1Apparatus

K.4.1.1.1 Scissors

K.4.1.1.2 Petri dish (9 cm in size consisting of 1.33 cm x 1.33 cm grids)

K.4.1.1.3 Wash bottle

K.4.1.1.4 Stereo zoom microscope

K.4.1.2 Procedure

The line intersect method is used to estimate the length of hyphae and roots. Root length is measured by dispersing roots against a grid of squares on the bottom of a tray. The roots are spread apart from one another over a grid in 2 mm to 10-mm depth of water. The eyes of the observer are cast along all the horizontal and vertical lines of the grid and intersects on roots across the horizontal and vertical lines is counted using a hand held click counter.

K.4.1.3 Calculation

The root length is calculated as follows:
Root length = No. of intersects x (11/14) x grid size

Where, 11/14 is a constant, and the size of the grid is the length of one side of one square of the grid

K.4.2 Clearing and staining root specimens - Clearing and staining procedures requires root samples that should be washed free of soil. It is important that KOH and staining solution volumes are sufficient for the number of roots being processed and that, roots are not tightly clumped together for uniform contact with solutions. To ensure uniform staining, the roots should be chopped in to smaller (1-2 cm) segments.

K.4.2.1 Procedure

Wash root specimens under running tap water thoroughly. Place them in beaker containing 5-10% KOH solution for about 15-30 minutes. The concentration of KOH and time of incubation of roots depend upon the age and tenderness of the roots. Pour off the KOH solution and rinse the roots well in a beaker using at least three complete changes of tap water or until no brown colour appears in the rinse water. Cover the roots with alkaline H_2O_2 at room temperature for 10 minutes or until roots are bleached. Rinse the roots thoroughly using at least three complete changes of tap water to remove the H_2O_2. Cover the roots with 1% HCl and soak for 3-4 min. And then pour off the solution. Do not rinse after this step because the specimens must be acidified for proper staining. Incubate the roots with staining solution (0.01% acid fuschin in lactoglycerol or 0.05% trypan blue in lacto phenol) and keep them for 12 hours for staining. Place the root specimens in glass petri plate /multi well plate for destaining for 12 hours (destaining solution is 1:1 solution of glycerol and lactophenol).
If clearing and staining is not possible immediately then fresh roots can be kept moist and stored at 5°C (for several days), or may be preserved in 50% ethanol for months in tightly sealed vials. Stain the roots subsequently for 12 hours and destain for 24 hours to allow excess stain to leach from roots.

Semi-permanent slides of stained roots can be made with polyvinyl-lacto glycerol (PVLG) mountant. For temporary slide the stained roots can be observed in plain lactoglycerol.

APPENDIX M
TEST FOR EFFECTIVE ROOT GROWTH

M.1 POT CULTURE TEST

For the purpose of 'Pot Culture Test' plant nutrition solution, preparation of solutions and procedure shall be same as described in **C.1.1** and **C.2**.

M.1.2. After three weeks of growth thin down the number of plants in each pot to four uniform plants or as suitable to the pot. At the end of three months, take one set of pot from control and inoculated series and separate the plants carefully from the soil under slow running water. Obtain the number, length and mass of the plant as a whole including branch, seeds if any and roots. At the end of three months, harvest the shoot system (depending on the crop). Dry at 60°Cfor48 hours and determine the dry mass. For the above purpose maintain a minimum of four replications or a maximum of 16 replications.

M.l.2.1 Better vigour of roots in inoculated seeds if established from the data when compared to the control, is the confirmation of effectiveness of ASI. If there is 10 percent increase in the plants over the dry mass of uninoculated control without nitrate, it may beconcluded that the culture is of required quality.

M.2 METHOD FOR ESTIMATING MPN COUNT FOR *AZOSPIRILLUM*

a) Add 100 g ASI sample to 900 ml of sterile distilled water.
b) Shake for 10 minutes on a reciprocal shaker.
c) Make tenfold dilution series.
d) Pipette 1 rnl of each dilution (from 10^{-1} to 10^{-7}) to each one of 5 replicates (5 tubes containing nitrogen free bromothymol blue semi-solid malate media for *Azospirillum*).
e) Begin by taking aliquot from the highest dilution and proceed down the series with same pipette
f) Collect the tubes from 10^5, 10^6 and 10^7 dilution level.
g) Incubate the inoculated series for 2 days at 30°C.
h) Check the characteristic sub-surface while particles (+ or -) in malate semi-solid medium, a change to dark blue colour of the medium.

APPENDIX N
DETERMINATION OF TOTAL POTASSIUM SOLUBILIZING CELL COUNT

N.1 APPARATUS

N.1.1 *Pipettes*, graduated, 1 ml and 10 ml.

N.1.2 *Conical Flasks*, 150 ml and 250 ml.

N.1.3 *Screw Capped Tubes*, 10 ml.

N.1.4 *Incubator*

N.1.5 *Petri Dishes*, clear, uniform, flat-bottomed.

N.1.6 *Hot Air Oven*

N.1.7 *Autoclave*

N.1.8 *pH Meter*

N.1.9 *Colony Counter*

N.1.10 *Hand Tally or Mechanical Counting Device*

N.1.11 *Magnifying Lens*

N.2 REAGENTS

N.2.1 Use a medium of the following composition for analysis of total viable count and contamination:

Mannitol	15.0 g
Yeast extract	3.0 g
Peptone	2.0 g
Agar	18.5 g
Trace element solution	1 ml
Distilled water	1 000 ml

N.2.1.1 *Trace Element Solution*

Sodium molybdate	0.20 g
Boric acid	0.28 g
Manganese sulphate	0.23 g
Copper sulphate	0.01 g
Zinc sulphate	0.03 g
Distilled water	1 000 ml

L-2.2 Use a medium of the following composition for studying zone solubilization in KMBI:

Glucose	5.0 g
Magnesium sulphate	0.005 g
Ferric chloride	0.1 g
Calcium carbonate	2.0 g
Potassium mineral	2.0 g (mica powder)
Calcium phosphate	2.0 g
Distilled water	1 000 ml

N.3 STERILIZING & PREPARATION PROCEDURE FOR PLATES

The method same as described in **B.3**

N.4 PREPARATION OF SERIAL DILUTION FOR CELL COUNT METHOD

N.4.1 Dispense 10 g of KMBI to 90 ml of sterile distilled demineralized water and shake for 10 minutes on a reciprocal shaker or homogenizer. Make serial dilutions up to 10^{-10} level. Pipette out 0.1 ml or suitable aliquots of 10^{-6} to 10^{-9} dilutions using sterile pipettes and deliver to the petri dishes containing set medium as described in **N.2.1**.

N.4.2 Spread the aliquots over the plate uniformly with a spreader or use droplet method. Invert the plates and promptly place them in the incubator at 28 ± 2°C for 3 days. Use 3 replicates of 10^{-5}, 10^{-6} and 10^{-7} level.

N.5 COUNTING

N.5.1 Count the colonies with the aid of magnifying lens under uniform and properly controlled, artificial illumination. Use a colony counter, equipped with a guide plate and rules in centimeter square. Record the total number of colonies with the hand tally. To distinguish colonies from dirt, specks and other foreign matter, examine doubtful objects carefully.

N.5.2 Count all plates but consider for the purpose of calculation plates showing more than 30 and less than 300 colonies per plate. Disregard colonies which absorb congo red and stand out as reddish colonies. *Fraturia aurentia* (KMBI) stands out as white-opaque glistering and domed colonies. Count such colony numbers and calculate figures in terms of per litre of carrier. Also check for freedom from contamination at 10^{-5} dilution.

APPENDIX P
METHOD FOR ESTIMATION OF POTASSIUM SOLUBILIZATION ZONES

P.1 Take 10 g of KMBI in 90 ml sterile distilled water.

P.2 Make a ten folds dilution series up to 10^{10}.

P.3 Take 0.1 ml aliquot of 10^{-5} to 10^{-7} dilution using sterile pipettes and deliver to petri dishes containing potassium solubilization zone media.

P.4 Spread it uniformly. Invert the plate and incubate them up to 2 weeks at 28 ± 2 °C.

P.5 Count the colonies showing solubilization zones and measure their diameter. Calculate average zone of solubilization in mm.

APPENDIX Q
DETERMINATION OF NUMBER OF INDIVIDUAL MICROBIAL CELLS

Q.1 APPARATUS

Q.1.1 *Pipettes*, graduated, 1 ml and 10 ml.

Q.1.2 *Conical Flasks*, 150 ml and 250 ml.

Q.1.3 *Screw Capped Tubes*, 10 ml.

Q.1.4 *Incubator*

Q.1.5 *Petri Dishes*

Q.1.6 *Hot Air Oven*

Q.1.7 *Autoclave*

Q.1.8 *pH Meter*

Q.2 REAGENTS

Use a medium of following compositions for different microorganisms:

Q.2.1 Medium for enumeration of *Rhizobium*

Agar	20 g
Yeast extract	1 g
Mannitol	10 g
Potassium hydrogen phosphate (K_2HPO_4)	0.5 g
Magnesium sulphate ($MgSO_4.7H_2O$)	0.2 g
Sodium chloride (NaCl)	0.1 g
Congo red	2.5 ml
Distilled water	1 000 ml
pH	7.0

Q.2.2 Medium for enumeration of *Azotobacter*

Agar	20 g
Sucrose ($C_{12}H_{22}O_{11}$)	20 g
Ferric sulphate $Fe_2(SO_4)_3$	0.1 g
Dibasic potassium phosphate (K_2HPO_4)	1.0 g
Magnesium sulphate ($MgSO_4.7H_2O$)	0.5 g
Sodium chloride (NaCl)	0.5 g
Calcium carbonate ($CaCO_3$)	2.0 g
Sodium molybdate (Na_2MoO_4)	0.005 g
Distilled water	1 000 ml
pH	6.8 to 7.2

Q.2.3 Medium for enumeration of *Azospirillum* (Nitrogen Free Bromothymol Blue Medium)

Malic acid	5.0 g
Potassium hydroxide	4.0 g
Di-potassium hydrogen phosphate	0.5 g
Ferrous sulphate	0.05 g
Manganese sulphate	0.01 g
Magnasium sulphate	0.1 g
Sodium chloride	0.2 g
Calcium chloride	0.1 g
Sodium molybdate	0.002 g
Distilled water	1 000 ml
Bromothymol blue	2.0 ml
(0.5 Percent alcoholic solution)	
Agar	1.7 g
pH adjusted to	6.5 - 7.0

NOTE
Colour of the medium should be pale green.

Q.2.4 Medium for enumeration of phosphate solubilizing microorganisms

Glucose	10.0 g
Tri-calcium phosphate	5.0 g
Ammonium sulphate	0.5 g
Magnesium sulphate	0.1 g
Sodium chloride	0.2 g
Yeast extract	0.5 g
Manganese sulphate Trace	
Ferrous suIphate Trace	
Distilled water	1 000 ml
Agar	15.0 g
pH adjusted to	7 ± 0.2

Q.2.5 Medium for enumeration of potassium mobilizing microorganisms (Aleksandrov Medium)

Glucose	5g
Magnesium sulphate	0.005g
Ferric chloride	0.1 g
Calcium carbonate	2.0 g
Potassium alumino silicate (mica powder)	2.0g
Calcium phosphate	2.0 g
Distilled water	1 000 ml

Mix all the ingredients in about 750 ml of water, except glucose and autoclave. Filter sterilized glucose dissolved in 250 ml water is mixed with above solution after autoclaving.

Q.3 STERILIZING & PREPARATION PROCEDURE FOR PLATES

The method same as described in **D.3**

Q.4 PREPARATION OF SERIAL DILUTION FOR CELL COUNT METHOD

Dispense 30 g of consortia of microbial inoculants in 270 ml of sterile water and shake for 10 minutes on a reciprocal shaker. Make serial dilutions up to 10^{7} level. Pipette out 0.2 ml aliquots of 10^{5} to 10^{7} dilution and deliver it on the petri dishes containing set medium as described in **Q.2.1** on respective medium of each microorganisms to be enumerated, except *Azospirillum*. Spread the aliquots over the plate. Invert the plates and place them in the incubator at 28 ± 2°C for 7 days. Use 3 replicates of 10^{-5}, 10^{-6} and 10^{-7} level.
For *Azospirillum*, use the methods as described in Appendix **H**.

Q.5 COUNTING

Count total number of colonies on the plates/tubes as per the method described for each microorganism.

ANNEX A
(informative)
RECOMMENDATIONS RELATING TO DIRECTIONS FOR USE

A.1 Use before the expiry date and do not expose to direct sun light or heat.

A.2 This is not a chemical fertilizer, hence do not mix it or the inoculated planting material with chemical fertilizers or pesticides.

A.3 Use for the crops specified on the label.