F.No. 26-04/2022-CIR-I

भारत सरकार

Government of India

कृषि एवं किसान कल्याण मंत्रालय

Ministry of Agriculture & Farmers Welfare

कृषि एवं किसान कल्याण विभाग

Department of Agriculture & Farmers Welfare

वनस्पति संरक्षण, संगरोध एवं संग्रह निदेशालय

DIRECTORATE OF PLANT PROTECTION, QUARANTINE & STORAGE

केंद्रीय कीटनाशी बोर्ड ए वम पंजीकरण समिति

Central Insecticides Board and Registration Committee

एन. एच. 4, फरीदाबाद (हरियाणा)-121001

N.H. IV, FARIDABAD (HARYANA)-121001

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Dated: 23rd May, 2022

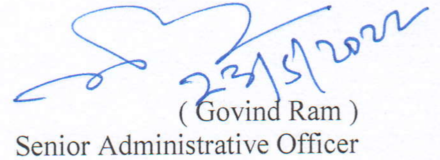
PUBLIC NOTICE

Subject-Guidelines for Registration of biopesticides along with consortium of Bio-pesticides-regarding.

The Registration Committee in its 439th meeting held on 25.04.2022 has approved the guidelines of Biopesticides along with consortium of Bio-pesticides under the Insecticides Act, 1968 proposed by the sub-committee constituted by the RC in its 427th meeting under the chairmanship of Dr. S.C.Dubey, ADG(PP &B), ICAR and Member RC for harmonization of data requirement for grant of registration of Bio-pesticides under the provision of IA,1968 after extensive consultation with the stakeholders.

Accordingly, the approved guidelines on Bio-pesticides are uploaded with this public notice for compliance by all stakeholders.

This issues with the approval of APPA & Secretary (CIB&RC).



(Govind Ram)
Senior Administrative Officer

Copy to:

1. Pesticides Associations.
2. Chairman, Registration Committee.
3. PPS to JS/ PPS to PPA.
4. IT Cell, HQ, Faridabad for uploading the same on the website.

GUIDELINES FOR REGISTARTION OF BIO-PESTICIDES

The RC in its 427th meeting constituted a subcommittee under the chairmanship of Dr. SC Dubey, ADG (PP & B), ICAR and Member RC for harmonization of data requirement for grant of registration of Bio-pesticides under the provision of IA, 1968. The guidelines for registration of Bio-pesticide have been prepared, discussed and comments of ICAR (NBAIR, NBAIM, NIBSM, IARI) have been received through ADG (PP). As per direction of 431st RC, the guidelines are finalized after discussion with toxicological experts for data requirement. Public notice was issued for comments of stakeholders on the draft guidelines on bio-pesticides and draft guidelines on consortium of bio-pesticides on dated 15.2.2022. Final Guideline is prepared after incorporating the suggestions of stakeholders if justified.

Bio-pesticides/ Microbial Pest Control Agents (MPCA)

1. The applicant needs to submit MOU/license agreement between the applicant and the inventor (either own R&D Laboratory or outsourced Research Institute/Facility) or Authorization letter from the inventor of strain OR undertaking by the applicant about the name of inventor/source of strain as per **Annexure-I.**
2. Undertaking declaring that the product is free from Chemical pesticides/Botanicals pesticides/Other Agro-Chemicals as **Annexure-II.**
3. Undertaking on bio-pesticides composition as **Annexure-III.**
4. Updated Stakeholder list for all members in Association/Organization claiming for MOU/authorization for data/technology utilization for mass multiplication/commercialization of the strain, **if applicable.**
5. Form-I dully filled and signed giving complete details along with requisite fee as applicable.
6. Notarized copy of BOD Resolution/ affidavit in case of proprietor/ partnership deed in case of partnership firms.
7. Correct composition as per earlier 9(3) / 9(3B) registrant of bio-pesticide strain.
8. The applicant should also, submit notarized copy of the Permanent Account No. (PAN), allotted by the Income Tax Department.
9. In case of company, the Certificate of Incorporation granted by the Registrar of Companies.
10. Fee receipt issued by NBAIM, Maunath Bhanjan, UP after submission of sample for DNA verification.
11. Undertaking that the product is free from GMO as **Annexure-IV**

The Data Requirements for Microbial Pest Control Agents (MPCA) Pesticides

i) Entomopathogenic/ Entomotoxic Bacteria
ii) Antagonistic Bacteria
iii) Entomopathogenic Fungi
iv) Antagonistic Fungi
v) Nuclear Polyhedrosis Virus (NPV) & Granulosis Virus (GV)

Chemistry:

Sl. No.	**Characteristics**	**Microbial (Antagonistic bacteria, Entomopathogenic/Entomotoxic bacteria, Entomopathogenic fungi, Antagonistic fungi, and Baculovirus**			
		Primary culture/mother culture		Formulated product	
		9(3B)	**9(3)**	**9(3B)**	**9(3)**
1.	Systematic name (Genus and species)	R	R	R	R
2.	Strain name	R	R	R	R
3.	Common name, if any	R	R	R	R
4.	Source of origin	R	R	R	R
5.	Specification of the product containing Habitat, Physical appearance and morphological description, pH, particle size, suspensibility, miscibility etc parameters	R	R	R	R
6.	Isolation of strain & Manufacturing process	R	R	R	R
7.	Methods of analysis including Quantitative analysis	R*	R*	R*	R*
8.	Shelf life claims	R	R	R	R
9.	Data on storage stability as per shelf life claims	NR	R	NR	R
10.	Composition of the product	R	R	R	R
11.	Potency of product by bioassay method (LC 50 (Beta, Delta , Cry toxin endotoxin content, classification (delta	NR#	NR#	NR#	NR#

	endotoxin)				
12.	CFU/g or ml	R	R	R	R
13.	POB/Capsule count pr ml/g of the product	R[%]	R[%]	R[%]	R[%]
14.	Adjuvants	NR	NR	R	R
15.	Human pathogens (culture method)	R	R	R	R
16.	Percent content of the Bio-control mass/organism in the formulation & nature of biomass.	R	R	R	R
17.	Percentage of carrier/filler, wetting/ dispending agent, stabilizers/ emulsifiers, contaminants/ impurities etc.	R	R	R	R
18.	Moisture content	R	R	R	R
19.	Contaminants: Pathogenic contaminants such as Salmonella, Shigella, Vibrio and such other microbials, not exceed 1 x 10^4 count per ml or per g of formulation	R	R	R	R
20.	Undertaking for free from Chemical and botanical pesticide contaminants	R	R	R	R
21.	Natural occurrence of the organism	R	R	R	R
22.	PCR / Immunology assays ELISA Test	NR[$]	NR[$]	NR[$]	NR[$]
23.	Separation and purification of crystals	NR	NR	NR	NR
24.	A sample for verification (500[@] g or 500[@] mL as the case may be)				
	a. DNA fingerprinting for the strain verification from Mau Bhanjan.	R	R	R	R
	b. Pre-registration verification at Central Insecticide laboratory (CIL)	R	R	R	R

* Test procedure and criteria used for identification – morphology, biochemistry, serology/ Immunology for Entomotoxic bacteria.

and $ these parameters are required for Entomotoxic bacteria.

% these parameters are required for Virus.

@ Two samples of same batch of 500 gm/ml each along with copy of the Fee receipt shall be submitted to Central Insecticides Laboratory, Faridabad for PRV purpose by the applicant for Entomotoxic bacteria.

- POB/Capsule count per ml/g of the product only for NPV.
- Viral unit: NPVs $1x10^9$ POB/ml or gm. minimum, GVs: $5x10^9$ capsules /ml or gm minimum.
- Dual culture to attain at least 50% reduction in target organism (35% for antagonistic bacteria). Bioassay: based on diseased severity and root colonization.
- Natural occurrence of the organism, Immunology assays: Elisa and Separation and purification of crystals are required for Entomotoxic bacteria.
- Test procedure and criteria used for identification by DNA test (Restriction enzymes analysis test).
- Biological assays for determining the LC_{50} / LD_{50} of the formulation for Entomopathogenic Viruses. Production of Entomopathogenic Viruses at commercial-scale was done exclusively *in-vivo* by culturing large number of larvae of host insect and subsequently feeding them with semi-synthetic diet contaminated with virus inoculums in laboratory. Viruses production *in-vitro* by culturing insect cells in bioreactors was a substitute for labour intensive maintenance of the massive host-insect colony.
- Manufacturing process including type of fermentation and biological end products. The microbial cultures are multiplied by liquid solid fermentation. Information pertaining to use of entire mycelia mats with spores separated must be provided in terms of biomass.

Documents to be mandatorily furnished by applicant applying u/s 9 (3)/ 9(3b) for all categories of bio pesticides

1. Verification of the Authorization letter submitted by the applicant via e mail by Secretariat from the original inventor/source of the strain for data utilization for mass multiplication.

2. MOU/license agreement between the applicant and the inventor (either own R&D Laboratory or outsourced Research Institute/Facility) or Authorization letter from the inventor of strain OR undertaking by the applicant about the name of inventor/source of strain as per **Annexure-I**

3. Updated Stakeholder list for all members in Association/ Organization claiming for MOU/authorization for data /technology utilization for mass multiplication/commercialization of the strain.

4. Relevant Affidavit/Undertakings: -
    a) Affidavit on bio-pesticide composition on NJSP duly notarized.
    b) Notarized copy of depositing microbial bio-pesticides strain sample in National Repository with reference code number.
    c) Undertaking on NJSP duly notarized that product do not contain any genetically modified organism in the prescribed format.

d) Undertaking on NJSP duly notarized that product is free from chemical /botanical pesticides / other agro-chemicals.
e) Affidavit of strain Innovator or applicant
f) Copy of 9(3B) Registration certificate, if relevant

Note:

1. Bt products should be labelled with bio potency and (or) toxin content. In addition, the labels will have to contain a measurement of toxin protein as percent protein, referring to the Lepidopteran-active toxin(s) present in the crystal.
2. The presently used Bt var. kurstaki standard is HD-1-S-1980 and its potency was calculated at 16,000 IUs per milligram of powder (Beegle et al. 1986. Standardization of HD-1-S-1980: US Standard for Lepidopterous-active *Bacillus thuringiensis*. Bulletin Ent. Soc. America 32: 44-45.). This standard strain is now available with PDBC, Bangalore and DOR, Hyderabad.
3. Defined potency and toxin concentration – Bioassay would require the use of an insect species. Normally manufacturers could select *Trichoplusia ni* / *Helicoverpa armigera* for Lepidopteran specific Bt formulations. *Spodoptera* Units (SPU), *Leptinotarsa* Units (LTUs) or International Toxin Units (ITUs) are to be used for denoting a specific insect.
4. No test for beta exotoxin is required for *Bacillus sphaericus*, because this species is not known to produce exotoxins.
5. The biopotency of products based on *B. thuringiensis* subsp. *israelensis* (*Bti*) is compared against a reference strain IPS82, 1884 using early fourth-instar larvae of *Aedes aegypti* (strain Bora Bora). The toxicity of IPS82 has an arbitrarily assigned toxicity of 15,000 ITU/mg powder.
6. The biopotency of products based on *B. sphaericus* (*Bsh*) is determined against a reference standard SPH88, strain 2362 using early fourth-instar larvae of Culex pippins (strain Montpellier). The toxicity of SPH88 has an arbitrarily assigned toxicity of 1,700 ITU/mg of the powder (Guidelines for laboratory and field testing of mosquito larvicides, WHO 2005 pp 45).
7. The use of alternative bacterial reference powders and / or strains must be approached cautiously. Such alternatives must be the subject of careful cross-calibration against the reference powders and should be conducted by recognized laboratories and should be made available to anyone who wishes to use, or check, the test with the alternative powders/strains.
8. Water content should not exceed 8 %, (12% in *Pseudomonas spp*) to preclude premature degradation of the product.

Guideline for already registered formulation/Strain u/s 9(3) under the IA, 1968.

Applicant shall submit only one folder containing the following documents:

I. Form-I dully filled and signed giving complete details along with requisite fee as applicable.

II. Notarized copy of BOD Resolution/ affidavit in case of proprietor/ partnership deed in case of partnership firms.

III. Correct composition as per earlier 9(3) / 9(3B) registrant of bio-pesticide strain.

IV. The applicant should also, submit notarized copy of the Permanent Account No. (PAN), allotted by the Income Tax Department.

V. In case of company, the Certificate of Incorporation granted by the Registrar of Companies.

VI. Authorization letter from the inventor of strain OR undertaking by the applicant about the name of inventor/source of strain as per **Annexure-I.**

VII. Requisite number of stamped envelopes.

VIII. Copies of Label Leaflets of the product as approved by RC of already registered strain.

IX. Copy of letter of Accession No. of strain or information on Accession number of strain.

X. Undertaking declaring that the product is free from Chemical pesticides/Botanicals pesticides/Other Agro-Chemicals as **Annexure-II**

XI. One sample of 500 gms/ml quantity shall be deposited to NBAIM, Maunath Bhanjan for test relating to DNA fingerprinting particularly partial gene code sequencing of desired strain and a fee may be paid directly to NBAIM through DD or online.

XII. Original fee receipt issued by NBAIM, Maunath Bhanjan, UP.

XIII. Undertaking that the product is free from GMO as **Annexure-IV**

XIV. Undertaking on bio-pesticides composition as **Annexure-III**

XV. Toxicology data shall be accepted from Non-GLP laboratory also for encouraging of new strain registration/any new or repeat studies for old strain. This decision shall be applicable to all categories of biopesticide registration, henceforth. The applications under scrutiny in the Secretariat of CIB&RC are also covered under this decision.

XVI. A sample of 500 gms/ml shall be deposited in the Secretariat of CIB&RC along with File/documents for PRV purposes.

XVII. The above folder shall be scrutinized by the Chemistry division of the Secretariat of CIB&RC.

XVIII. No preliminary scrutiny is required for applications for already registered strain of bio-pesticides.

XIX. A letter may also be written by the Secretariat of CIB&RC to the Director, NBAIM, Mau Nath Bhanjan, UP for submitting the DNA finger print report directly to the Secretariat of CIB&RC, certifying that the DNA of strain submitted by the applicant (Strain No.) matches with original Strain or otherwise.

XX. RC also decided that any government laboratory willing to undertake such studies on the terms and conditions as approved by the committee may request Secretariat of CIB&RC so as to seek approval from RC.

Annexure-I

AFFIDAVIT ON BIO-PESTICIDE STRAIN BY INVENTOR OR APPLICANT

I, …………………….. S/o ……………………………………, aged ……………years, resident of ……………………………………………..and Proprietor/Authorized person of the firm M/s …………………………………………………………., having its office at …………………………………………………….. do hereby declare and solemnly affirms as under:

That I am in the capacity of …………………………….. of firm M/s ……………………………… do hereby declare that the information furnished with respect to composition in Form-I, Label/Leaflet and bonafide verification of the application fir registration of (Name of the product)…………….., CFU/PBO……………per gm or ml min; Strain No. (Name and number of registered strain)…………. invented by M/s. …………………………is registered under section 9(3b) or 9(3) of the Insecticides Act, 1968.

1. That a shelf life of the product shall be twelve/Six/four months.
2. The product shall be packed as per IS: 8190 (Part-I) 1988 for Solid Pesticide (Second Revision).
3. That there will be no change in chemical composition, shelf-life, packaging requirement and the product will have the quality and packaging as per the relevant IS or as per specification approved by Registration Committee for 9(3b) & 9(3) registrant.

I, shall be responsible for adhering to the above composition and strain while manufacturing and marketing the product for distribution or sale. In case of any violation of the above declaration and also the conditions laid down on the Certificate of Registration of the said Bio-Pesticide, interalia, Product Quality Speciation submitted by us and also to the specification as and when the same are formulated and published by BIS amendments thereof, I am liable to be prosecuted/rejection of application under the provisions of Insecticide Act, 1968 and the Rules 1971 and amendments thereof.

Deponent

VERIFICATION

I, ……………………………., the above deponent do hereby verify that what has been declared above is true to the best of my knowledge and belief and nothing has been concealed there from.

Deponent

Annexure-II

UNDERTAKING FOR ABSENCES OF CHEMICAL/ BOTANICAL PESTICIDES/ CONTAMINATS/OTHER AGRO-CHEMICALS;

I, …………………….. S/o …………………………………, aged ……………years, resident of ………………………………………………..and Proprietor/Authorised person of the firm M/s ……………………………………………………………., having its office at …………………………………………………….. do hereby declare and solemnly affirms as under:

That the product, (Name of the product)……………………….. formulation containing CFU/PBO/Delta endotoxin content…………. per gm or ml min, Strain code, (if any)…………..Strain No: ……………. manufactured by (Name of the applicant)……………… does not contain any Chemical/Botanical Pesticide/Contaminants/other Agro-Chemicals.

That I/we shall provide the samples of our product (Name of the product) ……………………………. as and when desired by the competent Authorities of Government of India for verification.

That my/our above undertaking is true, and no portion is false and I have concealed nothing relevant to the above matter.

Place & date: Deponent Signature:

Name Designation:

Company Seal:

Annexure-III

AFFIDAVIT ON BIO-PESTICIDE COMPOSITION

I, …………………….. S/o …………………………………, aged …………….years, resident of ……………………………………………..and Proprietor/Authorised person of the firm M/s ……………………………………………………….., having its office at …………………………………………………….. do hereby declare and solemnly affirms as under:
That I am in the capacity of …………………………….. of firm M/s ……………………………… do hereby declare that the information furnished with respect to composition in Form-I, Label/Leaflet and bonafide verification of the application for registration of (Name of the product) ……………., CFU/PBO…………….per gm or ml min; Strain No. (Name and number of registered strain)………… under section 9(3b) or 9(3) of the Insecticides Act, 1968 is as under:-

1. COMPOSITION: (SPECIMEN FOR Pseudomonas fluorescence WP)

Components

A. Quantity (% w/w)
a) Pseudomonas fluorescence CFU 1x108 CFU/gm min 1.0%
b) Carboxy methyl cellulose 1.0%
c) Talc Powder98.0%
d) Total 100.0%

2. That a shelf life of the product shall be twelve/Six/four months.
3. The product shall be packed as per IS:8190 (Part-I) 1988 for Solid Pesticide (Second Revision).
4. That there will be no change in chemical composition, shelf-life, packaging requirement and the product will have the quality and packaging as per the relevant IS or as per specification approved by Registration Committee for 9(3b) registrant.
5. Bonafide declare that M/s ………………………………, manufacturing premises proposed/located at …………………………………………… having Registration Certificate total no., if any or Nil and manufacturing license no. if any or Nil.

I shall be responsible for adhering to the above composition while manufacturing and marketing the product for distribution or sale. In case of any violation of the above declaration and also the conditions laid down on the Certificate of Registration of the said Bio-Pesticide, interalia, Product

Quality Speciation submitted by us and also to the specification as and when the same are formulated and published by BIS amendments thereof, I am liable to be prosecuted/rejection of application under the provisions of Insecticide Act, 1968 and the Rules 1971 and amendments thereof.

Deponent

VERIFICATION

I, ……………………………., the above deponent do hereby verify that what has been declared above is true to the best of my knowledge and belief and nothing has been concealed there from.

Deponent

Annexure-IV

UNDERTAKING BY MANUFACTURERS OF MICROBIAL PESTICIDES

I,------------------------------------,aged-------years, s/o--, R/o--and--of M/s.---Registered Office at---do hereby undertake as follows:

(a) That the product---based on--------------------, Strain----------------------------, manufactured by M/s.-------------------------------- and /or imported by M/s……………………………………..does not contains any genetically modified organism (GMO) .

(b) That I/We shall abide by the provisions contained in the International Plant Protection Convention with regard to the import of this product.

(c) That I/We shall abide by the provisions in context of International Standards for Phyto-Sanitary Measures-Code of Conduct for the import and release of exotic bio-pesticides of the International Plant Protection Convention (IPPC), FAO, Rome. /Plant Quarantine (regulation of Import into India) order,2003.

(d) That I/We shall provide the samples of our product as and when desired by the competent authorities of Government of India for verification.

(e) That I/We further undertake that in the event of the above product having proved otherwise by any competent authority and resulting in environmental damage, I/We shall inform to Plant Protection Adviser, Dte. of PPQ&S, Sectt. of Central Insecticides Board and Registration Committee, and other relevant authorities for Manufacturing Licensing, Pollution Control and of appropriate District/State/National Level and shall comply with the directions from them.

(f) That my/our above undertaking is true, and no portion is false and I have concealed nothing relevant to the above matter.

Signature:----------------

Date------- Name--------- ----------

Place:---------------- Designation---------------Seal of the Company- ----

Bio-efficacy

Sr. No.	Particulars	Primary culture/mother culture		Formulated product	
		9(3B)	9(3)	9(3B)	9(3)
1	**Field studies:** Data on bio-effectiveness and phytotoxicity generated at ICAR, SAUs, CSIR / ICMR institutes. The data should be certified either by the Director or Head of the Institute.	NR	NR	R **	R***
2	**Laboratory studies:** The product should be tested at a laboratory under ICAR/ SAU/ CSIR/ICMR. **2.1)** LC50 values for each insect species under laboratory conditions should be generated at least at two institutes of ICAR, SAUs, CSIR and ICMR. **2.2)** Data on LC50 values for each target insect species should be generated at a laboratory under ICAR/ SAU/ CSIR/ICMR	R	R	R	R
3	Data on non-target organism: One season/one year on effect on product against natural parasites/ predators.	NR	NR	R	R

R = Required, NR = Not Required

R - Two seasons/years data on bio-effectiveness from two agro-climatic Zones**

R * - Two seasons/years data on bio-effectiveness from minimum three agro climatic Zones.**

2.1) - Applicable for Entomotoxic Bacteria
2.2 – Applicable for NPV & GV.
Sr. No. 3 - Required in case of Entomopathogenic fungi, Entomopathogenic Bacteria.

Note: No bio-efficacy data required for already registered strains of Bio-pesticides. Certificate of Registration will be granted as per approved formulation u/s 9(3)

Toxicity

S.No.	Parameters	Microbial (Antagonistic bacteria, Entomopathogenic/Entomotoxic bacteria, Entomopathogenic fungi, Antagonistic fungi, and Baculovirus			
		Primary culture/mother culture		Formulated product	
		9(3b)	9(3)	9(3b)	9(3)
1.	**Single Dose Oral – Rat** (Toxicity/Infectivity/Pathogenicity)	R	R	R	R
2.	**Single Dose Dermal – Rabbit** (Toxicity/Infectivity/Pathogenicity)	R	R	R	R
3.	**Acute Inhalation (a)**	R	R	R	R
4.	**Single Dose Pulmonary – Rat (b)** (Toxicity/Infectivity/Pathogenicity)	R	R	R	R
5.	**Single Dose Intraperitoneal – Rat (c)** (Toxicity/Infectivity/Pathogenicity)	R	R	R	R
6.	**Single dose intravenous (d)**	R	R	R	R
7.	**Primary Skin Irritation - Rabbit**	R	R	R	R
8.	**Primary Eye Irritation - Rabbit**	R	R	R	R
9.	**Skin Sensitization - Guinea pig**	R	R	R	R
10.	**Cell culture (d)**	R	R	R	R
11.	**Human Safety Records** (Effect/Lack of effects)	NR	R	NR	R
12.	**Toxicity to bird (1 species)** (Toxicity/Infectivity/Pathogenicity)	NR	NR	NR	R (Only 1 Species)
13.	**Toxicity to Fresh water Fish** (Toxicity/Infectivity/Pathogenicity)	NR	NR	NR	R
14.	**Toxicity to Honey bees (e)**	NR	NR	NR	R
15.	**Toxicity to Silkworm (f)**	NR	NR	NR	R
16.	**Toxicity to Earthworm (g)**	NR	NR	NR	R

Note:

a - Inhalation toxicity study required for registration of entomopathogenic/entomotoxic bacteria

b - Pulmonary toxicity study required for registration of antagonistic bacteria, antagonistic fungi, entomopathogenic fungi, baculovirus

c - Intraperitoneal toxicity study required for registration of antagonistic fungi, entomopathogenic fungi, antagonistic bacteria

d - Cell culture and Intravenous study required for registration of baculovirus.

e and f – required for all except antagonistic fungi

g- required for all except entomopathogenic/entomotoxic bacteria

Note: No data required for already registered strain from the same source with same strain designation and accession number

Note: If genome sequence of conserved region of the microbial strains/microbes used as microbial pest control agent is identical with already registered strain then data is not required from toxicity angle.

Formulations developed from similar already registered mother culture using similar ingredient and process of manufacture then no data is required from toxicity.

(I) The replacement alternatives not involving experiments on animals should also be given due consideration in case alternative if available with full and sound justification is provided specifically case to case basis.

(II) Waivers should be considered only when existing information provides robust and scientifically sound weight of evidence (WOE) approach, which will include examination of all the existing data of animal toxicity and other information/data (in chemico, in vitro, ex vivo, in vivo, in silico) and read across/bridging from structurally and/or biologically related similar bio-pesticide specifically case to case basis.

PACKAGING

Chapter V of the Insecticides Rules 1971 in the Insecticides Act, 1968, the rule 16 to 20 of the said chapter deals with the Packaging and Labelling.

Sl. No.	Parameter	Primary culture/mother culture		Formulated product	
		9(3B)	**9(3)**	**9(3B)**	**9(3)**
1.	**Labels and Leaflets as per IR-1971, all fields (as applicable) and as amended from time to time**	R	R	R	R
2.	**Manner of labeling and Leaflet**	R	R	R	R
3.	**Type of packaging (Ultra small, small or Big whichever is applicable)**	R	R	R	R
4.	**Manner of packaging**	R	R	R	R
5.	**Specification for primary, Secondary and Transport packages (whichever is applicable)**	R	R	R	R
6.	**Details of packaging material and its compatibility with content**	R	R	R	R
7.	**Performance of container with content during storage stability test(Shelf life Study)**	R*	R	R*	R
8.	**Transport worthiness test**	R*	R	R*	R

R*- Before Commercialization the data will be required.

Note:

1. In case of additional packaging endorsement applications, the data at Sl. No. 05, 06, 07& 08, are not required if similar packaging (material) is being sought by the applicant as has been granted to earlier 9(3) registrant.
2. Specification of Bureau of Indian Standard (BIS) must be followed for all the packaging requirements (Wherever available and applicable).
3. All Packaging tests must be carried out with the product of same batch and in its commercial package preferably in Indian condition.
4. The duration of the test and the conditions including geographical conditions must be mentioned.
5. Storage stability data must be generated keeping at least the following parameters in test protocol viz., temperature, duration, test packaging material, content of active ingredient in the product, test humidity, exposure to light (if applicable), physical and chemical properties of the product during and after storage etc.
6. The testing protocols must have their basis in the WHO/FAO/ CIPAC/ASTM recommendations or other validated methodology of GLP/ NABL accredited laboratory having packaging testing (chemical / mechanical as applicable etc.) in the scope.
7. The storage stability data for microorganisms can vary depending on the type of microbes. For Fungi, maximum storage stability study data will be 12 months at ambient temperature. For Gram negative bacteria like *Pseudomonas fluorescens* or other *Pseudomonas* species the maximum storage stability study data should be 8 months at ambient temperature. For spore forming gram positive bacteria like *Bacillus* species the maximum storage stability study data should be 18 months at ambient temperature.

   Note: Additional two months' data for six months self-life claim / three months additional data for one year and six months additional data for 18 months shelf-life claim at two/three different agro climatic locations at ambient temperature along with meteorological data should be submitted.

Besides this, the sub-committee also proposes the Guidelines on Consortium of Bio-pesticides.

Guidelines on Consortium of Bio-pesticides.

Efficiency of biocontrol agents could be increased by the development of mixture of compatible strains of different biocontrol organisms by considering the following norms. While developing a consortia formulation, the following needs to be addressed:

1. Compatible strains combination that differs in pattern of plant/site of colonization.
2. Compatible strains combination is broad spectrum of action against different plant pathogens.
3. Compatible strains combination with different modes of action under similar conditions.
4. Compatible strains combination of genetically diverse group to adapt to different pH, moisture, temperature and relative humidity.

The guidelines of Chemistry, Bio-efficacy, packaging for registration of consortia of Bio-pesticides are similar with the guidelines of Bio-pesticides except the following points.

Guidelines of mother culture/Primary culture of already registered bio-pesticides u/s 9(3) category are not required for registration of consortium Bio-pesticides. Only the guidelines of formulated product (Consortium) will be required. **Ratio of each strain in the formulation is required.**

1. Following toxicology guidelines for consortia of Bio –pesticides is required.

Note: a -Inhalation toxicity study required for registration of entomopathogenic/entomotoxic bacteria

b -Pulmonary toxicity study required for registration of antagonistic bacteria, antagonistic fungi, entomopathogenic fungi, baculovirus

c -Intraperitoneal toxicity study required for registration of antagonistic fungi, entomopathogenic fungi, antagonistic bacteria

d - Cell culture and Intravenous study required for registration of baculovirus.

e and f – required for all except antagonistic fungi

g- required for all except entomopathogenic/entomotoxic bacteria

Note:

- If genome sequence of conserved region of the microbial strains/microbes which are used in consortia of Bio-pesticides to be used as microbial pest control agent is identical with already registered strain, then data is not required for mother culture but data is required for combination/consortia from toxicity angle.
- If any new formulation of microbes is made by using new ingredients with different processes of manufacture than data is required for the formulation.
- If any new combination/consortia /Mixture of microbial strains/microbe developed from already registered microbial strain than data is required only for the mixture and not for mother cultures from toxicity angle.

List of Fungi, Bacteria and Viruses for Consortia

1. Sl. No 591.*Trichoderma* spp *.(T. viride /T. asperellum, T. harzianum, T. virens etc.)*
2. Sl. No 589. *Gliocladium virensT. virens*,
3. Sl. No 679. *Ampyliomyces quisqualis,*
4. Sl. No 683. *Coniotyrium minitans,*
5. Sl. No 692. *Chaetomium globosum* and
6. Sl.No 693. *Aspergillus niger* (non-pathogenic/biotype)
7. Sl. No 588. *Bacillus subtilis*,
8. Sl. No 590 *Pseudomonas fluorescens, P. protegens, P. entomophila*,
9. Sl. No 677, *Streptomyces griseoviridis,*
10. Sl. No 678 *Streptomyces lidicus,*
11. Sl. No 684 *Agrobacterium radiobacter* K84.
12. Sl. No 592. *Beauveria bassiana*,
13. Sl. No 593. *Metarrhizium anisopliae*
14. Sl. No 594. *Nomuraea rileyi*, (New name: *Metarhizium rileyi*)
15. Sl. No 595. *Verticillium lecanii,* (New name: *Lecanicillium lecanii*)
16. Sl. No 675. *Verticilium chlamydosporium*, (New name: *Pochonia chlamydosporium*)
17. Sl.No.689. *Paecilomyces lilacinus,* (New name: *Purpureocillium lilacinum*)
18. Sl.No.718. *Myrothecium verrucaria-nematicide*
19. Sl. No 326. *Bacillus species* (includes *Bacillus sphaericus (syn: Lysinibacillus sphaericus)*, *Bacillus thuringiensis var. galleriae, Bacillus thuringiensis var. israelensis, Bacillus thuringiensis var. kurstaki, Bacillus thuringiensis var. tenebrionsis, Bacillus thuringiensis var. sandiego*, *Bacillus thuringiensis* var. *tolworthi* and *Bacillus albus*.
20. Sl. No 596 Granulosis Viruses (GV)
21. Sl. No 597 Nuclear Polyhedrosis Viruses (NPV) (includes *Spodoptera litura* NPV, *Spodoptera frugiperda* NPV, *Heicoverpa armigera* NPV, *Spodoptera mauritia* NPV, *Mythimna separata* NPV,

1. INDIAN STANDARDS ANTAGONISTIC FUNGI DRAFT SPECIFICATIONS

1. Form and appearance
2. pH
3. Composition

3.1 CFU/g of the product

3.2 Percent content of the Biocontrol organism in the

3.3 formulation & nature of biomass.

3.4 Percentage of carrier/filler, wetting/ dispending agent,

3.5 stabilizers/ emulsifiers, contaminants/ impurities etc.

3.6 Moisture content

4.0 CFU counts: *Trichoderma* $2x10^6$CFU/ml or gm. (Stability at 30°C and 65%RH).

5.0 Contaminants:

5.1 Biological Contaminants:

5.1.1 Pathogenic Contaminants: such as gram negative bacteria *Salmonella, Shigella, Vibrio* etc.: **absent**

5.1.2 Other contaminants should not exceed $1x10^4$/ml or g

5.2 Chemical/ botanical pesticides contaminants: **absent.**

6. Method of analysis:

6.1 CFU counts by serial dilution and examination under regular compound research microscope with bright field optics.

6.2 Plating for contaminants on specific media

6.3 Antagonistic mycolytic capability on target organism by bioassay on plants (Laboratory test).

6.4 Bioassay procedure based on diseased severity and root colonization as detailed in Appendix-I

Bioassay for plant disease antagonists based on disease severity and root colonization.

The target pathogen to be tested against has to be grown in Sand maize medium. The Sand-maize medium is prepared by adding sand 90g, maize 10g. and water 10ml in a saline or any glass bottle of 300ml capacity and then autoclaved twice. Then 5 mycelial discs of the test pathogen are transferred into the bottle and left for incubation for 15 days. Once the culture has grown well, the sand maize medium is mixed along with the fungal growth and 1g from this preparation is used as the inoculum after adjusting the CFU to 1 x10/g by addition of sand.

The plastic cups (5-6 cm diameter) filled with soil and FYM (3:1) have to be used. In each cup the filling should be done upto ¾th level. The pathogen inoculum is mixed with sand has to be applied upto 2cm depth in the plastic cups.

The bio-efficacy of the bio-agent shall be tested by both seed treatment and soil application. For seed treatment, the recommended dose of the formulation has to be used (5 to 10g.). For soil application, the bio-agent is added at the rate of 1g of formulation (minimum CFU should be the $2x10^6$). The germination percentage, disease intensity and seedling vigour are to be recorded.

Another set of plastic cups filled with sterile soil and sterile FYM has to be used to confirm whether the bio-efficacy was due to the isolate of the bio-agent tested or due to the native isolates of the bio-agent present in the soil.

The keys for grading the efficacy mentioned below shall be used (Srivastava et al., 2002). However, for the registration purpose, the bio-agents that are Highly Efficient, Efficient or Moderately Efficient in the plastic cup test under glass house condition (in the presence of pathogen) can be allowed (i.e.) germination percentage of 70% or above, disease incidence of 30% or less can be considered for registration.

Disease Grading Key

Disease incidence (%)	Description	Rating of bio-efficacy of bioagents
0	Germination>90%, no seed rotting, seedling healthy, root and shoot portions well developed	Highly Efficient (HE)
1-15	Germination 80-90%, infection on main as well as lateral roots, seedlings are well developed	Efficient (E)
16-30	Germination 70-80%, development of roots restricted and growth is less compared to Score 1. Infection occurred on roots. Shoot portions developed but growth retarded compared to Score 1.	Moderately Efficient (ME)
31-45	Germination 60-70%, length of roots and shoots short compared to Score 1. Germination of seeds inhibited. 50% of root area infected. Shoot portions also showed infection	Moderately Inefficient (MI)
46-60	Seed germination 50-60%. Development of roots and shoots greatly retarded. Shoot portions also showed infection.	Efficient (I)
Above 60	Less than 50% germination and seed rotting	Highly Inefficient (HI)

For the root colonization assay, the rhizosphere region of the plants tested above have to be collected and the soil adhering to the root surface has to be removed by gently tapping the roots. The root bits have to be cut into 1 cm bits and randomly 25 bits should be selected for each treatment. They have to be plated on (TSM) and the percentage of root bits colonized has to be recorded. This has to be performed in the sterile soil and non-sterile soil. One control treatment without the Biocontrol agent, being tested, should be kept for both the sterile and non-sterile soil to rule out of the possibility of interference of native micro flora in the bio-efficacy assay.

2. INDIAN STANDARDS ANTAGONISTIC BACTERIA DRAFT SPECIFICATIONS

1. Form and appearance
2. pH
3. Composition

3.1 Percent content of the Biocontrol organism in the formulation & nature of biomass

3.2 CFU/g or ml of the product.

3.3 Percentage of other components: carrier /filler, wetting/ dispersing agent, stabilizers/emulsifiers, contaminants/impurities etc.

3.4 Moisture content.

3.5 CFU counts: Minimum $1x10^8$ CFU/ml or gm. (Stability at 30°C and 65%RH).

4. Contaminants:

4.1 Biological Contaminants:

4.1.1. Pathogenic Contaminants: such as gram negative bacteria

Salmonella, Shigella, Vibrio etc.: **absent**

4.1.2 Other contaminants should not exceed $1x10^4$/ml or g

4.2 Chemical/botanical pesticides contaminants: **absent.**

5. Method of analysis:

5.1 CFU counts on specific medium.

5.2 Plating for contaminants on specific media

5.3 Antagonistic capability on target organism by bioassay.

5.4 Bioassay procedure based on diseased severity and root colonization as detailed in Appendix-I

APPENDIX- I

Bioefficacy assay for plant disease antagonists based on disease severity and root colonization:

The pathogen to be tested against has to be grown in sand maize medium. The sand-maize medium is prepared by adding sand 90g, maize 10g and water 10 ml in a saline or any glass bottle of 300ml capacity and then autoclaved twice. Then 5 mycelial discs of the test pathogen are transferred into the bottle and left for incubation for 15 days. Once the culture has grown well, the sand maize medium is mixed along with the fungal growth and 1 g from this preparation is used as the inoculum after adjusting the cfu to 1 x 10/g by addition of sand.

The plastic cups (5-6 cm diameter) filled with soil and FYM (3:1) have to be used. In each cup the filling should be done upto ¾th level. The pathogen inoculum is mixed with sand has to be applied upto 2 cm depth in the plastic cups.

The bio-efficacy of the bio-agent can be tested by both seed treatment and soil application. For seed treatment, the recommended dose of the formulation has to be used (5 to 10g). For soil application, the bio-agent is added at the rate of 1g of formulation (minimum cfu should be the 2 x 10^6, the CIB recommended dose). The germination percentage, disease intensity and seedling vigour are to be recorded.

Another set of plastic cups filled with sterile soil and sterile FYM has to be used to confirm whether the bio-efficacy was due to the isolate of the bio-agent tested or due to the native isolates of the bio-agent present in the soil.

The keys for grading the efficiency mentioned below can be used here (Srivastava et al., 2002). However, for the registration purpose, the bio-agents that are Highly Efficient, Efficient or Moderately Efficient in the plastic cup test under glass house condition (in the presence of pathogen) can be allowed (i.e.) germination percentage of 70% or above, disease incidence of 30% or less can be considered for registration.

Disease Grading Key

Disease incidence (%)	Description	Rating of bio-efficacy of bio-agents
0	Germination>90%, no seed rotting, seedling healthy, root and shoot portions well developed	Highly Efficient (HE)
1-15	Germination 80-90%, infection on main as well as lateral roots, seedlings are well developed	Efficient (E)
16-30	Germination 70-80%, development of roots restricted and growth is less compared to Score I. Infection occurred on roots. Shoot portions developed but growth retarded compared to Score I.	Moderately Efficient (ME)
31-45	Germination 60-70%, length of roots and shoots short compared to Score I. Germination of seeds inhibited. 50% of root area infected. Shoot	Moderately Inefficient (MI)

	portions also showed infection	
46-60	Seed germination 50-60%. Development of roots and shoots greatly retarded. Shoot portions also showed infection.	Inefficient (I)
Above 60	Less than 50% germination and seed rotting	Highly Inefficient (HI)

For the root colonization assay, the rhizosphere region of the plants tested above have to be collected and the soil adhering to the root surface has to be removed by gently tapping the roots. The root bits have to be cut into 1 cm bits and randomly 25 bits should be selected for each treatment. They have to be plated on TSM and the percentage of root bits colonized has to be recorded. This has to be performed in the sterile soil and not sterile soil. One control treatment without the biocontrol agent being tested should be kept for both the sterile and non-sterile soil to rule of the possibility of interference of native microflora in the bio-efficacy assay.

For the bacterial antagonists, the above bioassay procedure has to be followed where only the % root colonization will be considered and other parameters are not required. The % root colonization required is 80%.

3. INDIAN STANDARDS ENTOMOPATHOGENIC FUNGI DRAFT SPECIFICATIONS

1. Form and appearance
2. pH
3. Composition

3.1 CFU/g of the product

3.2 Percent content of the Biocontrol organism in the formulation & nature of biomass.

3.3 Percentage of carrier/filler, wetting/ dispending agent, stabilizers/ emulsifiers, contaminants/ impurities etc.

3.4 Moisture content

4. CFU counts: Minimum $1x10^8$ CFU/ml or gm. (Stability at 30ºC and 65%RH).

5. Contaminants:

5.1 Biological Contaminants:

5.1.1 Pathogenic Contaminants: such as gram negative bacteria Salmonella*, Shigella, Vibrio* etc.: **absent**

5.1.2 Other contaminants should not exceed $1x10^4$/ml or g

5.2 Chemical/botanical pesticides contaminants: **absent.**

6. Method of analysis:

6.1 CFU counts by serial dilution and examination under regular compound research microscope with bright field optics.

6.2 Plating for contaminants on specific media

6.3 Entomopathogenic capability on target insects by bioassay.

Laboratory bioassay procedures for screening fungal pathogens on *Spodoptera litura* and *Helicoverpa armigera*

Insect pathogens:

Beauveria bassiana, Metarhizium anisopliae, Nomuraea rileyi

Preparation of Fungal inoculum for bioassays:

The fungus is grown on SDAY/SMAY medium for 10 days in slants and aqueous spore suspensions of various concentrations are prepared using sterile water. The spore count is estimated by Haemocytometer. (10^4 - 10^{10} spores/ml). Tween-80 is added @ 0.01% to get uniform spore suspension.

Rearing insects:

H.armigera, S.litura - Artificial diet (Semi-synthetic diet)

Stage of insect for bioassay

H.armigera, S.litura - II instar larvae to be used for bioassay protocols for lepidopteron pests

Method of inoculation

S. litura

1. Cut castor leaf discs of 3.0cm diameter, rinse in sterile distilled water and place each leaf disc in a sterile Petri plate and allow it air dry in a laminar flow system

2.Apply ten micro liters of the spore suspension of each concentration on the leaf disc and spread it uniformly on the leaf surface and allow it air dry in a laminar flow system. Treat the other side of the disc similarly.

3. Release ten numbers of second instar larvae of S. litura on the leaf surface and incubate the discs in an incubator at 25^0C and 90% RH

4. After 24hours, shift the larvae to the polypots containing the semi-synthetic diet and incubate in an incubator at 25^0C and 90% RH

5. After 5 days of incubation, mortality of the larvae are recorded in each concentration tested

6. Lc-50 can be calculated using SPSS package

Standard for LC-$_{50:}$ Not more than $2.00X10^6$ spores/ml ($3.0X10^3$ spores/mm^2)

H. amigera:

Instead of castor leaves, soybean leaves can be used for ***H. amigera*** and the procedure is same as above.

Standard for LC$_{50:}$ Not more than $4.00X10^6$ spores/ml (6.0X103 spores/mm^2)

Appendix-II

Bioassay procedure for *Plutella xylostella*

Various concentrations of *Beauveria bassiana* formulation ranging from 6 x 10^8 to 2 x 10^{10} are to be screened to assess the mortality.

Fresh undamaged radish leaves free from pesticide application are to be collected and washed thoroughly in sterile distilled water and air-dried. Individual leaves are dipped in respective concentrations for 30 seconds. After complete drying of leaves ten late 2nd instar larvae of *Plutella xylostella* are released per treatment. A water dipped radish leaf is maintained simultaneously as control.

To prevent desiccation of leaves, the petiole is covered with a moist cotton swab. Each treated leaves are placed in a plastic container of dimension 12.5 x 10 cm containing moist filter paper, Whatman No.41 to provide humidity.

Each treatment has to be replicated thrice. Fresh radish leaves were provided as feed at 24 hours interval. This set up has to be maintained at 25+1^0C and 70-80% RH for 7 days. Observations on larval mortality are to be made at 3, 5 and 7 days after treatment.

Standard for LC_{50} = Not more than 3 x 10^9 cfu/g

4. INDIAN STANDARDS, ENTOMOTOXIC BACTERIA TECHNICAL /FORMULATION DRAFT SPECIFICATIONS

S.No.	Details
1. SCOPE	
1.1	This Indian Standard prescribes the requirements and the method of sampling and test for Entomotoxic bacteria technical and formulation. The product is a biopesticide active against target insects. The product is not for human consumption.
2. REQUIREMENTS	
2.1	Common name: i.e., *Bacillus thuringiensis or B. sphaericus etc.*
2.2	Systematic name (Genus, species, serotype, strain and Cry-toxin* along with cry gene)
2.3	Physical specification 2.3.1 Form and appearance 2.3.2 Moisture content 2.3.3 pH
2.4	Composition 2.4.1 Delta endotoxin content (Minimum 2.0%) – estimation as per Appendix-V 2.4.2 Adjuvants 2.4.3 Beta Exotoin content – Negative through housefly bioassay test as per Appendix-IV 2.4.4 Human pathogens (gram negative bacateria Salmonella, shigella & vibrio etc) - Absent 2.4.5 Other microorganisms (not more than 10^4 / g) 2.4.6 Chemical/botanical pesticide contamination - Absent
2.5	Natural occurrence of the organism
	2.5.1 Its relationship of the organisms 2.5.2 History (exotic or indigenous strain) 2.5.3 The isolate should not be genetically modified organism (GMO).
3. SAMPLING	
3.1	Representative samples of the material shall be drawn in accordance with IS 10946:1984
4. TESTS	

4.1	An appropriate test procedure and criteria used for identification, such as morphology, biochemistry and / or serology / immunology 4.1.1 Morphology description, particle size 4.1.2 Immunology assays: ELISA / Dot blot assay test or any other sensitive standard immunology test. 4.1.3 Method of analysis 4.1.4 Level of beta exotoxins to be identified if expressed by Housefly bioassay method. 4.1.5 Potency of product by bioassay method (**Appendix-II**) 4.1.5.1 Bioassay method a) LC 50 on target larvae and potency against a reference using artificial diet or leaf disc method or in the water for mosquito larvae (Appendix-I) b) Housefly Bioassay method for Beta-exotoxin content (Appendix-IV) c) Determination of toxin content by ELISA / Dot Blot Assay Method (Appendix-V) 4.1.5.2 A technique for the separation and purification of the crystals (Appendix III) is to be used by the manufacturer and the antisera to be raised using solubilized toxin. Toxin content (3.5 %) to be standardized in the formulation using this antisera (ELISA /Dot blot assay).

2.2 Crytoxin* If H-Serotype is not known, it is mandatory to provide the details of Cry toxin to confirm that it is *Bacillus thuringiensis.*

Appendix I

Bioassay Method

Diet incorporation

The following protocol is used for diet incorporation of oral toxicants to test their toxicity on target insects. The example presented here is to bioassay Cry I Ac on *H. Armigera* (First instar larva of other test insects are used for similar bioassay).

1. Pipette out 3 ml of the solution into a 40 ml plastic cup.
2. Pour lukewarm diet, approx. 60^0 C, into the cup to a total volume of 30 ml. Place the lid and shake the cup vigorously for a minute to mix properly.
3. Pour the diet to 0.5 cm height, into wells of a 24-cell insect- rearing tray. Allow the diet to cool in laminar airflow under UV lamps for I h to surface sterilize the diet.
4. If concentration of the toxicant in the stock solution was 2 μg/ml, the final concentration in the diet would now be 0.2μg/ml diet. Thus the final concentration of toxin in diet was diluted 10-fold.
5. Release first instars into the diet rearing trays at the rate of one per well. Cover the diet tray with semi-permeable wrap and close the lid.
6. It is recommended that the lid be tightly secured to the tray with rubber bands, to prevent the larvae from escaping. Because the diet is unsuitable, larvae try constantly to escape from the diet rearing trays.
7. Keep controls with larvae released on untreated diet, for all the experiments.
8. The unused rearing trays with diet can be stored in a refrigerator for a week.
9. Change the diet for the larvae every two or three days.
10. Record mortality observations at 8 hourly intervals until the end of seven days, for median lethal time LT_{50} calculations. LT_{50} is the time at which 50 % of' the test population is killed with the specific dose tested. A simple linear regression equation can be worked out to calculate the LT_{50}
11. Otherwise, record mortality at alternate days until the end of seven days, for median lethal concentration LC_{50} calculations. LC_{50} is the concentration that kills half the test population.

I2. Record weights of surviving larvae at the end of seven days, for median effective concentration EC_{50} and LC_{50} is the concentration that prevents half the test population from reaching 50% of the weight attained by control larvae. For example, if the average weight of larvae on the control diet (without toxin) was 80 mg, EC_{50} represents the concentration at which 50% of the test population is unable to gain a weight more than 40 mg. LC_{50} is the concentration that inhibits half the test population from reaching the third instar.

Diet incorporation for filter paper bioassays

1. For bioassays with bollworms, 10 ml toxin incorporated diet is poured over a16 sq cm piece of filter paper. The filter papers layered with diet are cooled and cut into smaller squares of 2 x 2 cm, and 10 first instar larvae are released in small plastic cups 3 x 3 cm (d x h) cups containing a square. Change the strips every alternate day.

2. Record mortality observations until the seventh day.

Surface coating of semi-synthetic diet

I. Prepare the diet and pour it into the trays or the rearing plastic cups. Generally, 10 µl of the toxin can be used to coal 1 sq cm surface area. Gently swirl the diet surface to ensure uniform and complete spread of the solution over the diet surface.
2. Allow the surface to dry in a laminar airflow under UV light for 2-3 hours to surface sterilize.
3. Release one first instar *H. armigera* larva per well. Always maintain proper controls with untreated diet.
4. Change the diet on alternate days and record mortality until the seventh day. Then, weight of surviving larvae should be recorded on the final day of the bioassay.

The method has the advantage of obtaining constantly reliable results because the toxin is unlikely to be affected by either improper mixing or heat as can occur in the diet-incorporation method. Moreover, less amount of the toxin is required for the assay, compared to the diet-incorporation method.

Calculation of results:

The potency of the sample (International Units – IUs)

$$\textbf{IU/mg sample} = \frac{\text{LC50 Standard}}{\text{LC50 Sample}} \times \text{IU/mg Standard}$$

(IU/mg Standard, i.e., HD-1-S-1980 is 16,000 IUs/mg; the US standard is available with PDBC, Bangalore; each registrant should prepare a "self reference" and should deposit it with the Registering Authority. Each self reference will be expressed as IU/mg using International standard)

Exotoxin determination by PCR studies

Methodology:

Some *Bacillus thuringiensis* strains secrete type I β- exotoxin, which is a non-specific insecticidal and thermostable adenine nucleoside oligosaccharide. Toxicity bioassays and HPLC are traditional methods for detecting β-exotoxin. For rapid approach for prediction of type I β-exotoxin production, PCR-based method can be followed as per Diego H Sauko *et al*. (2014). One of these ORFs encodes the Exo protein that was proved to be responsible for the phosphorylation of a β-exotoxin precursor at the last step of their biosynthesis process (Liu et al., 2010). Primers BEF (forward; 50- CGGCAGCCGTTTATTCAAA-30) and BER (reverse; 50-CCCCTTCCCATGGAGAAACA-30) amplify a 406-bp DNA fragment of thuE between nucleotides 373 and 778. All *B. thuringiensis* strains are grown on nutrient agar plates for 16h. A loopful of cells is transferred to 100 µl H_2O and boiled for 10 min to make DNA accessible for PCR amplification. The lysate is centrifuged briefly (5 s at 20,000g), and 5 µl supernatant is used as a DNA template in each polymerase chain reaction. This is performed with a final volume of 25 µl containing 2.5 µl 10x reaction buffer, 0.5 µl 50 mM MgCl2, 0.5 µl 100 mM deoxynucleoside triphosphate mixture, 8 pmol each primer, and 1 U of Taq polymerase (Invitrogen). The PCR amplification consisted of DNA denaturation at 94°C for 2 min followed by 25 cycles of amplification with a gradient thermocycler. Each cycle consisted of a denaturation step at 94°C for 1 min, an annealing step at 54°C for 1 min, and a chain elongation step at 72°C for 1 min. The final elongation step was extended for an additional 5 min. Subsequently, 10 µl PCR product is analysed by 1.0% agarose gel electrophoresis. A positive control can be also used for better results.

Appendix-II

Dot Blot assay of *Bacillus thuringiensis* (B.t.) toxin protein as alternate of Bioassay.

1) B.t. grown till sporulation in shake flask or in fermenter vessel and let the cells lyse and release spore/crystals into the medium

2) Cells are harvested by centrifugation at 10k for 15 mins.

3) Wash the pellet with 1M NaCl to remove the B.t. associated seine/metallo proteases and washd twice with sterile distilled water.

4) Pellet suspended in 50MM NaOH to solublize the toxin protein for 2 hours at R.T. with slow shaking and centrifuged again at 10K for 15 Mins.

5) Supernatant was adjusted to pH 8.0 with Tris HCL pH 8.8

6) Protein contents estimated by Lowry's protocol.

7) Two fold serial dillutions of test protein were made in PBS and known amount at protein applied on NCP using S&S or Biorad Dot Blot manifold apparatus and applying water vaccum for 30 mins.

8) NCP was carefully removed from Dot Blot set and soaked in excess of 3% Skim milk in PBS for blocking the remaining acetic sites on NCP for 2-3 hours at R.T/O/N at 4^oC.

9) Wash the NCP with excess PBS with 0.01% Tween 20, 3-4 times and then finally with PBS

10) Polyclonal antiserum raised against total crystal protein was suitably diluted in PBS and added to the 'seal a meal' containing NCP and incubated for 1-2 hours with shaking.

11) Remove the NCP from the bag and was several times (as mentioned in step.No.9)

12) Anti-rabbit antibodies conjugated with HRPO/alkaline Phosphate was diluted as per the suppliers instruction and incubated NCP (as in step 10)

13) Was as in step 11

14) For HRPO:

    a) Diaminobenzene (4mg/10ml PBS)/4-Chloro-1-Napthol (4mg/10ml 20% Alcohol) were dissolved and 10ml of 30% of H2O2 per 10 ul substrate soluion was added and colour reaction developed in dark for 5-10 mins (DAB gives brick red colour. 40N gives blue colour).
    b) For alkaline Phosphatase:

Alkaline Phosphatase Buffer:

1M Tris pH 8.8 - 10ml/
4M NaCI - 2.5ml/ make up to 100ml
1M Mgc12 - 0.5ml/

For 10ml of above buffer add NBT-66 ul and BCIP-33 ul and developed and colour reaction

15. Stop the reaction by removing the substrate and washing with PBS.
16. Keep on filter paper and dry.

DIFFERENT PROTEIN CONCENTRATION

10ug 5ug 2.5ug 1.25ug 512.5ng 256.25ng 128ng 64ng 32ng 16ng 8ng 4ng

Determination if cell dry weight

Take a known volume of Bacterial culture spin down at 4R for min.

Wash the pellet in minimal distilled water

Transfer to a pre weighed container

Incubate at 80^0C for 16-18 hours till become dry and weight becomes constant.

Appendix-III

PURIFICATION OF CRYSTALS BY GELATIN METHOD

Centrifuge the sporulated material and wash pallet twice with 1M Nacl. Add 200ml. of 0.5% Gelatin, stir and remove all froth completely. Dilute with sterile water and centrifuge. Take debris and stir with 20ml. of 1.5M sucrose. Further add 50 ml of 1.5M sucrose, stir and centrifuge at 3000 RPM for 2 hours. Remove supernatant and purified crystals are harvested.

5. INDIAN STANDARDS BACULORIVUS DRAFT SPECIFICATIONS

1.Form and composition of the product

1.1 Viral Unit: POB/Capsule count per ml/g of the product

1.2 Percent content of the bio-control organism in the formulation and nature of biomass

1.3 Percent of carrier/filler, wetting/dispersing agent, stabilizers/ emulsifiers, containments/ impurities etc.

2. Moisture content

3. pH

4. Viral Unit:

NPVs (*Helicoverpa & Spodoptera*) - $1x10^9$ POB/ml or gm (minimum) (POB –Polyhedral Occlusion Body)

GV (*Chilo, Plutella & Acheae*) - $5x10^9$ Capsules/ml or g. (minimum).

5. **Contaminants**:

5.1 **Biological contaminants**:

5.1.1 **Pathogenic contaminants**: Pathogenic contaminants such as gram negative bacteria *Salmonella, Shigella, Vibrio* etc. should be **absent:**

5.1.2. **Other microbial contaminants:** Other microbial contaminants should not exceed $1x10^4$ /ml or g

5.2 Chemical/botanical pesticides contaminants should be absent**.**

6. Identification of Baculovirus by DNA test (Restriction enzyme analysis test).

7. An undertaking should be submitted that the strain is indigenous, naturally occurring and not exotic and not genetically modified as per Annexure-1.1

8. **Method of analysis:**

Viral Unit:

NPVs (*Helicoverpa and Spodoptera*) $=1x10^9$ POB/ml or gm. minimum

GVs = $5x10^9$ Capsules/ml or gm. minimum.

8.1 In case of NPVs/, POB/Capsule count should be taken with Haemocytometer using shallow depth counting chamber as detailed in Appendix – I

8.2 Biological assay for determining the LC_{50} or LD_{50} of the formulation:

8.2.1 Bioassay for NPV by the Diet Surface Contamination Method as detailed in Appendix-II

OR

8.2.2 Bioassay for GV against *Chilo infuscatellus* as detailed in Appendix-III OR

8.2.3 Bioassay for GV against *Plutella xylostella* as detailed in Appendix-IV.

8.2.4 Bioassay for GV against *Acheae janta* as detailed in Appendix-V.

8.3 Plating for contaminants on specified media.

COUNTING OF NPV/GV (POB/CAPSULE) USING IMPROVED NEUBAUER HAEMOCYTOMETER COUNTING CHAMBER.

A haemocytometer is used for estimating of NPVs/GVs in a unit volume of the product. The Improved Neubauer Haemocytometer comprised a thick glass slide with a shallow depression in the central section divided into two halves (figure-1). Each side, the base of the depression has a fine ruled grid of squares (figure-2) which is visible under a microscope. The dimensions of this grid are defined. Place a standard cover slip placed over the depression and a one half halves of the slide chamber using a micro pipette. The particles require 2-5 minutes to sediment to the chamber floor.

Either dark field or a phase contrast microscope is used to identify and count polyhedral occlusion bodies (POB) or capsule. With the counting chamber under the microscope, the number of Polyhedra/capsule in a given number of grid squares can be counted. Each count consists of a tally of the number of polyhedra completely contained within a big square plus the number of touching the top and left sides. Polyhedra touching the bottom and right sides are not counted. Since both the depth of the chamber and the grid dimensions are known. It is then a straight forward calculation to determine the number of polyhedra /capsule per ml of test suspension.

$$\text{Number of NPV (POB) per ml/gm} = \frac{D \times X}{N \times K}$$

Where:

D = Dilution factor

X = Total number of polyhedra counted

N = Number of squares counted

K = Volume above one small square in cm^3 =($2.5x10^{-7}cm^3$)

Area of each small square is 1/400 mm^2 = 0.0025 mm^2. Depth of chamber is o.1mm. Volume of liquid above a single small square is 0.0025 mm^2 x 0.1mm= 0.00025 mm^3. To covert to cm^3 multiply by 1/1000 to get a volume of 2.5×10^{-7} cm^3 above 1 small square. Hence, $K=2.5x10^{-7}cm^3$

Worked example:

Suppose in a sample diluted by a factor of 1000 we count 535 polyhedra in 160 small squares then:

$D = 1000$

$X = 535$

$N = 160$

$K = 2.5 \times 10^{-7}\ cm^3$

$$\text{Thus, POB count} = \frac{1000 \times 535}{160 \times 2.5 \times 10^{-7}} = 1.34 \times 10^{10}\ \text{POB/ml of test sample}$$

Note: (i) Usually, this procedure is repeated 3 times and an average taken to get a more accurate estimate.

(ii) Same procedure will be used for GV also for counting the number of capsule per unit volume of the product.

PROCEDURE FOR ESTIMATION OF LC_{50} OF NPV BY THE DIET SURFACE CONTAMINATION METHOD.

i) **Diet to be used**: The standard chickpea-based diet without formaline.
ii) **Bioassay bottles**: 5ml. vials with a diameter of 18 mm (255 mm^2 surface area)
iii) **Doses of NPV to be tested**:

Helicoverpa armigera		*Spodoptera litura*	
POB/ml	POB/mm^2	POB/ml	POB/mm^2
a)$5x10^4$	1.96	$1x10^6$	39.21
b)$1x10^4$	0.39	$2x10^5$	7.84
c) $2x10^3$	0.078	$4x10^4$	1.57
d)$4x10^2$	0.016	$8x10^3$	0.31
e)$0.8x10^2$	0.003	$16x10^2$	0.062
f)1.6x10	0.0006	$3.2x10^2$	0.013

iv) **Method of dosing**: Dispense 10 Microlitre aliquots into each vial and spread uniformly over the entire diet surface using a polished rounded lip of 4 mm glass rod and allow to dry off under flow laminar hood for 10 minutes.

v) **No. of larvae/dose**: 50 (Maintain 50 healthy larvae without virus inoculation for control)

vi) **Stages of larvae**: II instar larvae (Preferably 4 days old)

Release one larva/vial and plug mouth with sterile absorbent cotton.

Incubate at 25 ± 1°C for 7 days.

vii) Record mortality in different doses on the 7th day.
viii) Apply Abott's formula for correction of mortality in control treatment.
ix) Subject the dose – mortality response to probit analysis using relevant statistical software.
x) Express LC_{50} as POB/mm^2 of diet surface.

Expected standards for NPV for II instar larvae

Species	LC_{50} POB//mm^2
1. *Heliocoverpa armigera*	< 0.5
2. *Spodoptera litura*	< 20.0

Bioassay for GV against *Chilo infuscatellus*

Determination of LD_{50}:

To determine the LD_{50} of the GVs, third instar larvae should be used. The larvae are to be microfed (one micro litre per larva) with six different doses, viz. 1.1 x 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 IBs/larva. One hundred freshly moulted larvae have to be used for each treatment. Larvae fed with equal quantity of distilled water serve as control. The mortality has to be recorded daily. The LD_{50} of the virus is determined following the probit analysis method (Finney, 1962).

$LD_{50} = < 1x10^3$ OB for third instar larvae by micro-feeding.

Laboratory bioassay procedures for estimation of LC_{50} of *Plutella xylostella* (PxGV) by leaf disc method:

1. Cut leaf discs of cauliflower (3.2cm). Soak it in 0.1N NaOCI for 5 min. and wash thoroughly in distilled water. Air dry these leaf discs for 2-3 minutes. (Fifth leaf from top to be used)
2. PxGV (containing 0.01per cent Triton X 100) of different concentrations 28000, 2800, 280, 28, 2.8 OB/mm^2 on the leaf disc) is prepared
3. Aliquots of 12ul of each concentrating of GV is dispensed on the upper surface of the leaf disc and spread uniformly with a blunt end glass rod (use separate tips and glass rods for each treatment)
4. Air dry these leaf discs for 2-3 minutes
5. Repeat the same on the lower surface of the leaf disc.
6. Control discs were treated with distilled water containing 0.01 per cent Triton X 100 only.
7. The leaf discs are placed in Petri dishes lined with wet filter paper discs and 35-second instar larvae of *P. xylostella* (starved for 6 hours) are released on each leaf disc starting from control treatment to highest concentration. This is replicated three times.
8. Incubate these larvae at 25^oC
9. After 24 hours remove the treated leaves (partially eaten) and provide the larvae with fresh cauliflower leaves.
10. The leaves are changed daily and mortality data recorded every day.
11. The dosage and time mortality responses are subjected to probit analysis.
12. If the mortality in the control excess 10% repeat the experiment.

LC_{50} = < 0.15 OB/mm^2 for second instar larvae by disc method.

Laboratory bioassay procedures for estimation of LC_{50} of *Achaea janata* Granulosis virus (AjGV) by leaf disc method:

1. Cut leaf discs of castor (8cm dia) and wash in distilled water. Air dry these leaf discs for 5 minutes.
2. Treat the leaf disc on both the upper and lower surfaces with 200 µl suspension of AjGV (containing 0.02% Tween-80) of different concentrations ($5x10^8$, $5x10^7$, $5x10^6$, $5x10^5$, $5x10^4$ corresponding to 19884, 1988, 198, 19, 1.9 OB per mm^2 on the leaf disc)
3. Aliquots of 100 µl of each concentration of GV is first dispensed on the upper surface of the leaf disc and spread uniformly with a blunt end of glass rod (use separate tips and glass rods for each treatment)
4. Air dry these leaf discs for 5 minutes
5. Repeat the same on the lower surface of leaf disc
6. Control leaf discs were treated with distilled water containing 0.02% Tween-80 only
7. The leaf discs are placed in Petri dishes (9.0cm dia) line on wet filter paper discs and 35 second instar larvae (third day after hatching) of *A. janata* are released on each leaf disc starting from control treatment to highest concentration. This is replicated three times.
8. Incubate these larvae at 25°C.
9. After 24-48 hours remove the treated leaves (partially eaten) and provide the larvae with fresh castor leaves
10. The leaves are change daily and mortality data recorded every day
11. The dosage and time mortality responses are subjected to probit analysis
12. If the mortality in the control exceeds 10% repeat the experiment.

Recommended LC_{50} GV (*Achaea janata) – LC_{50}* <4 OB/mm^2 for second instar larvae by the leaf disc method.