

**Registration of Bio-stimulants
under Schedule VI of FCO**

**Guidelines/Data requirement
for grant of Registration
under
various categories**

Chapter 1: General Guidance to the Applicants/Stakeholders

1. Stakeholders generating data and submitting the application for registration should ensure that the tests are conducted and data generated in accordance with established scientific procedures following the test guidelines and the principles of Good Laboratory Practices and recommended agricultural practices, as per the requirement. The data should be authentic, replicable, utilizable and of good quality. The complete study reports should be submitted.
2. The requirement for registration usually includes data and information on proposed application; data on identity of the Biostimulants (identity, composition, analysis and quality); data to assess risk to humans and the environment; data to assess efficacy of the product; its packaging (as per the material used by the applicant) and labelling requirements (contents to be defined from time to time). As per the Fertiliser (Inorganic, Organic or Mixed) (Control) Amendment Order, 2021, Schedule VI, section 20c, the manufacturer should give data on A) Chemistry, B) Bio-efficacy Trials, and C) Toxicity. Data on A) Chemistry and B) Bio-efficacy Trials are necessary for each of the product for its registration. In case of “toxicity” data requirement, Central Biostimulant Committee (CBC) may examine case to case basis based on the history of safe use, and/or bio-safety data of the product accepted by Government bodies of India or any developed Countries for its use in agriculture. Considering the diverse nature of biostimulants, the guidelines specified in the Fertiliser (Inorganic, Organic or Mixed) (Control) Amendment Order, 2021 should be followed.
3. The data requirement for registration of Biostimulants varies with the type of Biostimulant to be registered; the type of material to be registered; solid (WP, granules, powder) etc. liquid (EC, EW, SC etc.) or Vapor, if any (vaporizer, fumigants etc., if any); the category of registration – i.e. provisional or subsequent as per the provisions of the FCO, 2021; and purpose of registration – domestic use or export or for both (domestic use and export); the intended use of the Biostimulant to be registered or its label claims etc. Hence, before starting data generation or submitting application for registration, the applicant should ensure that the requirements are being complied correctly for the type of Biostimulant to be registered under the desired category and for the intended purpose.
4. The data generated via recognized Scientific Research Organizations and/or Industry Associations in supervision of recognized Research Organizations (SAUs, ICAR,

CSIR, etc for agronomic efficacy; NABL Accredited/GLP Laboratory for Chemistry Data generation) for a particular bio-stimulant product by following accepted procedures and protocols can be used by multiple manufacturers through legally acceptable terms and conditions. The product equivalence shall be maintained by the manufacturers.

5. In general, Bio-efficacy data needs to be generated through replicated trials conducted at minimum of three agro-ecological locations under National Agricultural Research & Education System comprising of ICAR Institutions, CAUs, SAUs and other recognized organizations and institutions. The doses (Control; optimum = 1x; 0.5x; 1.5x; 2.0x etc), number of treatments and other test parameters can be decided in consultation with the institutions by following established protocols. The complete data set, at the experimental site, generated for Bio-efficacy tests including environmental data, fertilizers used, irrigation schedule, agro-chemicals used, soil physical and chemical parameters, etc should be recorded, analysed, interpreted and submitted for drawing conclusions.
6. For Label Expansion Claims, data waivers based on established scientific justifications may be considered on case to case basis.
7. The product composition once accepted under Schedule VI, can be used for manufacturing the product after seeking necessary approvals.
8. For Toxicity Data Generation, existing literature and information should be analysed in a robust weight-of-evidence approach (WoE approach) prior to undertaking animal tests. Animal testing needs to be avoided where ever possible and waivers may be granted, if justified on the basis of scientifically sound rationale and literature published in reputed journals/reports. Scientifically validated in-vitro methods of safety evaluation can also be accepted to reduce experimental animal usage. Similarly, toxicity data on single species each of birds and fishes can be accepted.
9. Shelf life studies should be conducted as per the standard testing protocols and norms. If such protocols are not defined, the applicant should provide the verifiable methodology adopted.
10. No fortification of bio-stimulants with any other nutrients or chemicals shall be allowed. The naturally occurring beneficial elements limits/levels, which results during extraction process, can be considered on case to case basis.
11. No fortification of pesticides shall be allowed. The maximum permissible limit of pesticides in the biostimulants, which can ingress during the manufacturing process, is notified separately.

12. Tolerance limits for naturally occurring phyto-hormones shall be defined on case to case basis, based on the availability of such phyto-hormones in the raw natural products from which the bio-stimulant is being derived.
13. The data, if any, submitted by the applicant at the time of seeking provisional registration shall not be required to be resubmitted again at the time of submission of application for regular registration by the same applicant, if composition and other claims remain unchanged. In general, data submission for provisional registration is not required, but for inclusion in FCO, complete data set including bio-efficacy and toxicity data along with other documents is required to be submitted.
14. Standard designs, as prescribed from time to time for agro-chemicals, bio-fertilizers etc, shall be followed for packaging of biostimulants.

Chapter 2: Biostimulant categories, sub-groups and active ingredients

Unlike plant growth regulator and other agrochemicals, the exact mode of action and active ingredients are not clearly known in case of biostimulants. Further, depending upon the crop, stage of application, mode of application, soil-type and agro-ecological situations, the minimum limit of concentration required to bring out bio-efficacy will differ. Hence, it is difficult to define minimum and maximum limits/concentrations of specific active ingredient(s) in the product formulated for marketing under each category.

Table 1 indicates different categories of biostimulants, sub-groups of each category and description of category and active ingredients in each category of biostimulants (The list can be further expanded with the discovery of any other active ingredient).

Table 2.1: Biostimulant categories, sub-groups and active ingredients

S.No.	Biostimulant Category	Subgroups	Description of the category with active ingredients
1	Botanical extracts, including seaweed extracts	Botanical extracts	Extracts from plants (belonging to the kingdom plantae with five subgroups: 1) Thallophyta, 2) Bryophyta, 3) Pteridophyta, 4) Gymnosperms and 5) Angiosperms) Thallophyta are simplest form of plants with no differentiated plant body. Examples: red, brown and green algae Bryophytes include mosses (Funaria), Marchantia and liverworts. Pteridophytes (Vascular Plants) consist of plants with differentiated body into roots, stem and leaves. It has specialized tissue such as xylem and phloem for conduction of water and others from one to another part of the plant body. Examples: Marsilea, ferns and horse-tails. Gymnosperms (Coniferophyta) are vascular non-flowering plants that produce seeds without cover i.e., seeds are not enclosed in an ovary. Examples: cycads, ginkgo, yews and conifers Angiosperms are flowering plants that produce covered seeds. The kingdom contains more than 250,000 species. Therefore, botanical extracts include from any of the plants belonging to the kingdom Plantae. The molecular composition depends upon the species and genotype. <i>Active ingredients:</i> Several different molecules consisting of mixtures of primary and secondary metabolites and plant hormones.

		Seaweed extracts	Seaweed extracts are a sub-category of botanical extracts. Seaweeds are plants belonging to subgroup of plantae called Thallophyta. It includes Red (<i>Rhodophyceae</i>), brown (<i>Phaeophyceae</i>) and green (<i>Chlorophyceae</i>) algae. Composition of biostimulant depends upon the species from which it was prepared, and can also vary to some extent with the culture conditions used for cultivation of seaweeds. <i>Active ingredients:</i> Several different molecules consisting of mixtures of primary and secondary metabolites, polymers and plant hormones.
2	Biochemicals	Specific biomolecule and their combinations	Biochemicals are the molecules that are synthesized/produced in the metabolism of the living organisms. These naturally occurring biochemicals can also be synthesised by chemical synthesis in laboratories. <i>Active ingredients:</i> One or more biochemicals and their combinations
3	Protein hydrolysates and amino acids	Protein hydrolysates	Protein hydrolysates are mixtures of amino acids and peptides obtained from chemical or biological break-down of proteins from plant and animal sources. The peptide size may vary from 2–20 amino acid units but may be longer also. <i>Active ingredients:</i> free amino acids and polypeptides
		Amino acids	An amino acid is a carboxylic acid-containing an aliphatic primary amino group in the α position to the carboxyl group, and an organic R group (or side chain) that is unique to each amino acid. The word “amino acid” is derived from <i>α-amino [alpha-amino] carboxylic acid</i>. The following 20 amino acids that are building blocks of proteins in plants and animals: 1. Alanine; 2. Arginine; 3. Asparagine; 4. Aspartic Acid; 5. Cysteine; 6. Glutamic acid; 1. Glutamine; 8. Glycine; 9. Histidine; 10. Isoleucine; 11. Leucine; 12. Lysine; 13. Methionine; 14. Phenylalanine; 15. Proline; 16. Serine; 17. Threonine; 18. Tryptophan; 19. Tyrosine; 20. Valine Plants also produce non-proteinogenic amino acids (NPAAs) such as ornithine, citrulline, arginosuccinate, homoserine, homocysteine, cystathionine, pipecolic acid, γ-aminobutyric acid (GABA), canavanine, 1-aminocyclopropane carboxylate (ACC), etc. Canavanine is known to be toxic to animals,

			hence will be included in negative list and maximum limit in the bio-stimulant shall be disclosed by the applicant. Normally plants and animals produce L stereoisomers. Chemical synthesis often leads to production of mixture of L and D stereoisomers, which then are separated later. Some D-Amino acids may be toxic, hence will be included in negative list and maximum limit in the bio-stimulant shall be disclosed by the applicant. <i>Active ingredients:</i> One or more Amino acids including NPAAAs.
4	Vitamins (The specific vitamin listed are the active ingredient in each subgroup)	Coenzyme Q10 (CoQ10)	CoQ10 is 2,3-dimethoxy, 5-methyl, 6-decaprenyl benzoquinone, anisoprenylated benzoquinone and is also known as ubiquinone or ubiquinone-10. It is abundant in microbes, plants and animals. Involved in electron transport in mitochondrial and chloroplast electron transport chain. It is a lipid soluble antioxidant.
		Vitamin A	Fat-soluble; This group includes retinol, retinal (also known as retinaldehyde), retinoic acid, and several provitamin A, carotenoids (β-carotene); IUPAC ID of Retinol: (2E,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethylcyclohexen-1-yl)nona-2,4,6,8-tetraen-1-ol. Plants are rich source of β-carotene, the pro-vitamin A.
		Vitamin B1 (thiamine)	Water-soluble; Function as coenzyme in essential factor in central carbon metabolism. It is produced by all plants and most microbes. Thiamine (C₁₂H₁₇N₄OS) is 2-[3-[(4-amino-2-methylpyrimidin-5-yl)-methyl]-4-methyl-1,3-thiazol-3-ium-5-yl]ethanol). It is an organo-sulfur compound comprising pyrimidine and thiazolium heterocycles linked by a methylene bridge. This group consists of Thiamine, TMP (Thiamine monophosphate), TDP (Thiamine diphosphate), TTP (Thiamine triphosphate), ATDP (Adenosine thiamine diphosphate) and ATP (Adenosine thiamine triphosphate).
		Vitamin B2 (riboflavin)	Riboflavin is 7,8-dimethyl-10-(1'-d-ribityl) isoalloxazine. Water-soluble; Function as coenzyme; Riboflavin is a precursor for the coenzymes, flavin adenine

			dinucleotide (FAD) and flavin mononucleotide (FMN), which are mostly involved in redox reactions. It is produced by all plants and most microbes.
		Vitamin B3 (niacin)	Water-soluble; Function as coenzyme; IUPAC ID: pyridine-3-carboxylic acid ($C_6H_5NO_2$). It consists of niacin and niacinamide, also known as nicotinic acid (NA) and nicotinamide (Nam), respectively. Vitamin B3 are biosynthetic precursors to nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$), and play key role in intermediary metabolism, mitochondrial respiration (Electron acceptor in electron transport chain and the Krebs' cycle), reactive oxygen species generation and inhibition, and post-translational protein modifications, protein regulation and second messengers' generation.
		Vitamin B5 (pantothenic acid)	Water-soluble; Function as coenzyme; Pantothenic acid is the precursor of the 4'-phosphopantetheine moiety of coenzyme A and acyl-carrier protein. It is synthesized in plants and microbes. It is a dietary requirement for animals.
		Vitamin B6 (pyridoxine, pyridoxal, pyridoxamine)	Water-soluble; Function as coenzyme; It consists of six vitamins: pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM), and their phosphorylated derivatives. Pyridoxal-5'-phosphate (PLP) is a cofactor for over 140 biochemical reactions involved in amino acid, sugar, and fatty acid metabolism. Plants and microbes synthesize vitamin B6, but it is a dietary requirement for animals.
		Vitamin B8 (H-biotin)	Water-soluble; Function as coenzyme; Biotin is a coenzyme. It is covalently bound to a Lys residue of a group of enzymes that catalyze carboxylation, decarboxylation or trans-carboxylation reactions. Biotin containing carboxylases of plants are acetyl-CoA carboxylases, 3-methylcrotonyl-CoA carboxylase and geranyl-CoA carboxylase. Plants also contain a noncatalytic biotin protein that accumulates in seeds. IUPAC ID: 5-[(3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl]pentanoic acid.
		Vitamin B9 (folate)	Water-soluble; Function as coenzyme; Naturally occurring folates are dihydrofolate (DHF) and tetrahydrofolate (THF), and the later is ready to use form. Folate acts as donors and acceptors of one-carbon groups in one-carbon transfer reactions involved in

			biosynthesis nucleic acids, panthothenate (vitamin B5), amino acids, etc. Folates are synthesized in microbes and plants. But humans and other vertebrates are fully depending on their diet for folate supply.
		Vitamin B12 (cobalamin)	Water-soluble; Function as coenzyme; It is synthesized by certain bacteria and archaea. Plants and animals do not synthesize B12. The red algae <i>Porphyra</i> sp. and green alga <i>Chlorella</i> sp. produce biologically active B12. Vitamin B12 (cyanocobalamin) is a corrinoid that contains a corrin ring. Hydroxocobalamin, methylcobalamin, and 5'-deoxyadenosylcobalamin also act as coenzymes.
		Vitamin C (Ascorbic acid)	Water-soluble; Ascorbic acid (C₆H₈O₆); Plants synthesize Vitamin C but humans cannot. It is an antioxidant, and a cofactor of peptidyl-glycine alpha-amidating monooxygenase involved in the biosynthesis of many signalling peptides, such as oxytocin, vasopressin, cholecystokinin, and calcitonin. It is also a cofactor for many dioxygenases (reducing the iron in the active site of these enzymes to Fe²⁺), methylcytosine dioxygenases.
		Vitamin D	Fat-soluble; The two forms of vitamin D are cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2). Vitamin D2 is produced in fungi and yeast from ergosterol upon UVB-exposure, while vitamin D3 from 7-dehydrocholesterol in the skin upon UVB-exposure (290–315 nm). 7-DHC is also synthesized by some plants such as tomato, which can be converted to Vitamin D3 upon UV exposure.
		Vitamin E (α-Tocopherol)	Fat-soluble; It consists of tocopherols and tocotrienols; Vitamin E is synthesized solely by photosynthetic organisms. α-Tocopherol is the predominant form of vitamin E in green parts γ-tocopherol is the major in non-photosynthetic tissues of plants. α-tocopherol is a major scavenger of free radicals in the lipid bilayer and also transmits specific signals outside chloroplasts in plants.
		Vitamin K	Fat-soluble; It consists of Vitamin K1 (Phylloquinone) and K2 (menaquinone). Phylloquinone is a prenylated naphthoquinone synthesized by plants, green algae, and some cyanobacteria; It acts as an electron carrier in

			photosystem I and electron acceptor for the formation of protein disulfide bonds. Menaquinone is essential in electron transport and ATP generation in all Gram-positive, and anaerobic Gram-negative bacteria.
5	Cell free Microbials products	Cell free Microbials products	Extracts from microbes which are non-pathogenic and microbes that are not having bio-control activity. Consists of large number of different molecules. CFU should be NIL. <i>Active ingredients:</i> Several different molecules consisting of mixtures of primary and secondary metabolites and hormones
6	Antioxidants	Enzymatic antioxidants	Antioxidants are molecules that scavenge reactive oxygen species (ROS) such as superoxide and hydroxyl radicals. It can be classified in to enzymatic and non-enzymatic antioxidants. Total anti-oxidant capacity of such biostimulants can be determined by Trolox equivalence antioxidant capacity (TEAC) assay, ferric ion reducing antioxidant power (FRAP) assay, oxygen radical absorbance capacity (ORAC) assay, inhibiting the oxidation of low-density lipoprotein (LDL) assay, cellular antioxidant activity assay, etc. <i>Active ingredients:</i> Enzymatic antioxidants: SOD, Catalase, Peroxidase
		Non-enzymatic antioxidants	<i>Active ingredients:</i> One or more of the following molecules - Glutathione, polyphenols (phenolic acids, flavonoids, anthocyanins, lignans and stilbenes), carotenoids (xanthophylls and carotenes), vitamins [vitamin E (α-Tocopherol) and C (Ascorbic acid)], etc.
7	Anti-transpirants	Molecules that close stomata	Anti-transpirants are molecule that reduce transpiration through stomatal closure, increasing resistance to water vapor loss and/or reducing the heat load on leaf. <i>Active ingredients:</i> - Molecules that close stomata: Absciscic acid (ABA) Phenyl mercuric acetate (PMA)
		Film forming molecules that increase resistance to water vapor loss and increase	<i>Active ingredients:</i> One or more of the following molecules Chitosan; Waxes; Alumino-silicate (kaolin); Calcium carbonate (CaCO_3); Calcium oxide (CaO); di-l-p-menthene (pinolene); poly-l-p-menthene; Acrylic polymers; Potassium sulfate (K_2SO_4)

		reflectance	
8	Humic & fulvic acid and their derivations	Humic acids (HA) Fulvic acids (FA) Humin	Humic substances (HS) are components of the natural organic matter in soil, water and geological organic deposits such as lake sediments, peats, brown coals and shales. HS are formed by a process called humification consisting of biochemical and chemical reactions during the decay and transformation of plant and microbial remains. HS are polymers of heterogeneous molecules comprising of sugar, fatty acids and polypeptides as aliphatic chains and aromatic rings. HS are grouped in to three groups: humic acids (HA or HAs), fulvic acids (FA or FAs) and humin. HA are insoluble below pH 2, fulvic acids are soluble at any pH, and humin are insoluble in water. <i>Active ingredients:</i> humic acids (HA or HAs), fulvic acids (FA or FAs) and humin.

Chapter 3: Tolerance limit for major categories

1. Applicant/Manufacturer may list some major active ingredients in the product. The tolerance limit for these individual ingredients will be fixed at $\pm 5\%$ except for the biostimulant categories of botanical extracts including seaweed extract and Cell free Microbials products.
2. In the biostimulant categories of botanical extracts including seaweed extract and Cell free Microbials products, large number of molecules may act as biostimulant, and these molecules vary in their type, concentration and combination depending upon the species used for extraction of biostimulant. Further growth conditions used for cultivation of plants can also affect to significant extent by the concentration of individual biomolecules. Hence, for these categories the tolerance limit for these individual ingredients will be fixed at $\pm 10\%$ for individual active ingredient molecule.
3. Limits of Zinc and Copper included in heavy metals in the notification
 - The FAO/WHO recommends maximum permissible limit (MPL) of Zinc and Copper as 99.4 ppm and 73.3 ppm in the products consumed by humans. In the Fertiliser (Inorganic, Organic or Mixed) (Control) Amendment Order, 2021, the MPL permitted for Zinc and Copper is 1000 and 300 mg/kg of harvested products. The Maximum permissible level (MPL) of concentration of Zinc and Copper in Biostimulant formulation meant for foliar application prescribed by Regulation (EU) 2019/1009 is 1500 and 600 ppm, respectively.
 - For biostimulant products recommended for soil application, the limit of Zinc and Copper can be increased up to different recommended dose of these nutrients for soil application. The applicant /manufacturer shall submit in such cases, the recommendation of state department of agriculture/ Agricultural Universities/Institutes for the crops for which label claim of biostimulant is made.
 - The applicant/ manufacture should submit a data and certify that the concentration of Zinc and Copper in the final formulation is due to i) the extraction process and ii) concentration of the naturally present Zinc and copper in the source material from which the biostimulant is prepared, and iii) not intentional addition of these minerals in to a biostimulant preparation.
 - As per the definition of biostimulant, the efficacy of biostimulant should be due to other active ingredients not due to its nutrient content. Therefore, the agronomic efficacy trials, appropriate treatments (Comparison of biostimulants with the equivalent concentration of micronutrients present in the final product) should be included to prove that the beneficial effect is independent of its nutrient content *per se*.
4. Prescribed limits of B and Mo in different categories of bio-stimulants
 - Boron is an essential micronutrient for plant. About 0.7 ppm of boron in soil is optimal and more than 1.5 ppm may be toxicity for sensitive plant species. Molybdenum (Mo) is an essential micronutrient for plant and is required in very less amount. i.e. <0.2 ppm.
 - A concentration of 3–10 mg Mo kg⁻¹ dry matter of plant may be critical level beyond which it may cause toxicity to ruminants.

- Hence, a general recommendation of limits for these two micronutrients is unwarranted.
- In no case any micro-or macro-nutrient mineral elements should be added from exogenous source in any category of biostimulant. The CBC will examine on case-to-case basis in the event of micro-or macro-nutrient mineral elements from the endogenous sources are high in any biostimulant category and decide on the relaxation of limit.

Chapter 4: Generation of Data on eye irritation and skin irritation tests on rabbits

The eye irritation and skin irritation tests on rabbits will be conducted as per need. However, the CBC may consider exemption on case-to-case basis, if the manufacturer submit data previously generated for registration of the product elsewhere in the developed Countries and accepted by their regulatory agencies.

The data as on date from other regulatory agencies on the ‘No Observed Adverse Effect Level (NOEL) or LD50 or any toxicity relevant data for different biostimulants is indicated in Table 4.1.

Table 4.1: Information related to requirement of toxicity test for different Biostimulants

4.1.1. Botanicals

S.No.	Biostimulants	LD50mg/kg	NOAEL /LOAEL mg/kg/day (ppm)	Tox Profile		Global
				Symptoms	Effects	Regulations / Guidelines
1.	Botanicals Essential Oil: Eucalyptus, Blue Gum – Organic Essential Oil: Lemon, Distilled - Organic	Acute Oral (Rat) >2,480 mg/Kg Dermal (Rabbit) > 2,000 mg/Kg (2) Oral: >5 gr/kg (rat) Dermal: >5 gr/kg (rabbit)		Eye contact: May cause eye irritation and corneal damage if not immediately rinsed out. Skin Contact: Repeated contact may cause allergic dermatitis. Inhalation: Breathing high concentrations of vapor may cause anesthetic effects. (2) May be fatal if swallowed and enters airways.	Skin corrosion/irritation: May be irritating to skin. Redness of the skin if irritated. Serious eye damage/irritation: May be irritating to eyes. Prompt rinsing and removal of the substance will avoid damage. (2) (3) Respiratory or skin sensitization: Exposure to vapors from this solvent in excess of the stated occupational exposure limit may result in	Botanicals used in dietary supplements industry can have toxicology concerns related to endpoint gaps (1) EU See EU Allergens in Section 3 USA Not regulated by Federal or State Regulations California Prop. 65 Components This product contains chemicals

					adverse health effects such as mucous membrane and respiratory system irritation and adverse effects on kidney, liver and central nervous system. Aspiration toxicity includes severe acute effects such as chemical pneumonia, varying degrees of pulmonary injury or death following aspiration. (3) Biodegradation is expected. Avoid exposure to marine environments and waterways. (2) Known photosensitizer (3)	known to State of California to cause cancer, birth defects, or any other reproductive harm. No-observed-effect level (NOEL) and no-observed-adverse-effect level (NOAEL): use in animal health risk assessment (8) <i>Principles and Methods for the Risk Assessment of Chemicals in Food (2009)</i>, WHO Environmental Health Criteria, No. 240 have been adopted EFSA(7) & (9) Three step method by applying the weight-based classification
--	--	--	--	--	--	--

						to the NOEL, NOAEL and LOAEL based on the findings.(10)
References:						
1.	In silico approach to safety of botanical dietary supplement ingredients utilizing constituent-level characterization Jason G. Little, Daniel S. Marsman, Timothy R. Baker, Catherine Mahony Food and Chemical Toxicology 107 (2017) 418-429					
2.	SAFETY DATA SHEET (SDS) – eden botanicals Essential Oil: Eucalyptus, Blue Gum – Organic, SDS Created: June 2018 Revision Date: 27 May 2020					
3.	SAFETY DATA SHEET (SDS) – eden botanicals Essential Oil: Lemon, Distilled – Organic, SDS Created: July 23 2018 Revision Date: July 20 2021					
4.	BBC Biochemical MATERIAL SAFETY DATA SHEET Section 1. Chemical Product and Company Information Common Name: 10% Neutral Buffered Formalin					
5.	TOXICOLOGICAL PROFILE FOR BENZENE U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES Public Health Service Agency for Toxic Substances and Disease Registry, August 2007					
6.	TOXICOLOGICAL PROFILE FOR SELENIUM U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES Public Health Service Agency for Toxic Substances and Disease Registry, September 2003					
7.	European Food Safety Authority. (2017). Peer review of the pesticide risk assessment of the potential endocrine disrupting properties of glyphosate. EFSA Journal, 15, e4979. https://doi.org/10.2903/j.efsa.2017.4979					
8.	Australian Govt; Australian Pesticides & Vet Meds Authority, 2019					
9.	European Food Safety Authority. (2017). Quillaia extract/kg bw per day – NOAEL=400,750,1500					
10.	Toxicol. Res.: Vol. 27, No. 3, pp. 133-135 (2011) Open Access http://dx.doi.org/10.5487/TR.2011.27.3.133 A New Way in Deciding NOAEL Based on the Findings from GLP-Toxicity Test Yeong-Chul Park ¹ and Myung-Haing Cho ² GLP Center, Catholic University of Daegu, Kumrak 5-Ro, Hayang-Up, Kyongsan-Si, Kyongbuk 712-702, Korea ² Laboratory of Toxicology, College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea					

4.1.2: Seaweeds

S. No.	Biostimulants	LD50mg/kg	NOAEL /LOAEL mg/kg/day ppm	Tox Profile		Global Regulation / Guidelines
				Symptoms	Effects	
	Seaweeds	Acute toxicity (Annex IIA, point 5.2) Rat LD50		Skin irritation ‡ Non-irritant (3)The symptoms include	Inhalation may cause irritation of the nose, throat, and respiratory	Food Safety commission. Japan – Arsenic in foods (6)

		oral ‡ > 2000 mg/kg bw Rat LD50 dermal ‡ > 2000 mg/kg bw (2)		redness, itching and swelling. (9) Eye irritation ‡ Non-irritant(3) The symptoms may include redness, itching and tearing.(9) Skin sensitisation ‡ Non-sensitising (M & K) (2) Ingestion of large amounts may induce nausea.(3)Can be an irritant for the mouth, nose, throat and digestive tract if ingested.(9)	system(9) No exposure assessment was deemed necessary, as the substance does not present a toxicological concern. Exposure to consumers already exists, as sea-algae extracts are food-grade. Eco-toxicity: High concentrations may cause scorching in plants. Not toxic to vertebrates, invertebrates or marine algae.(3) Harmful if swallowed.(4) Dental disease was identified post-mortem in both the control and treated animals and was attributed to feeding high levels of pre-gelatinized waxy maize starch.(5) death in 15/20 rats (7)	
	PC 42 (4)	500 (4)				
	Carrageenan (5) (E 407) (8) Gardenia Yellow(5)	20 g/kg bw in mice and 4 g/kg bw in rats,	In infant baboons, a no-observed-effect level (NOEL) of 1220 mg/L in formula Maximum concentration of carrageenan in neonatal pig formula at the NOAEL is 2250 mg/L on mucosal mast cell counts. The NOAEL for carrageenan from this			
	Octenyl succinic acid (OSA)–modified starch (5) Pectin (5)	Acute oral 269 (LD50 in females)132 (LD50 adult) 220 (LD50 weanling) (7)				
	Barium (7)					

EU specification for E 407 in rats (8)

			study was 300 mg/kg formula, in GIT 3,400–3,900 mg/kg body weight (bw) per day, (8) (72 mg/kg bw per day(5) NOAEL of 2.5% pAOS (equivalent to 1.7 g/kg bw per day).(5) 66-198 mg/kg bw (rat) (exposure routes) 70-450 (mouse) (7)			
References:						
	1	TOXICOLOGICAL PROFILE FORPOLYCYCLIC AROMATIC HYDROCARBONS U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES Public Health ServiceAgency for Toxic Substances and Disease Registry (August 1995)				
	2	EFSA Journal 2012;10(1):2492 ; © European Food Safety Authority, 2012 CONCLUSION ON PESTICIDE PEER REVIEW Conclusion on the peer review of the pesticide risk assessment of the activesubstance sea-algae extract1 European Food Safety Authority, European Food Safety Authority (EFSA), Parma, Italy				
	3.	M ATERI AL SAFETY DATA SHEET Bio-Atlantis Eco-licitor ®Company & Product Identification Company Identifier: Manufacturer: Bio-Atlantis Ltd. Address: Kerry Technology Park, Tralee, Co. Kerry, Ireland.- OECD Environment Directorate				
	4.	Revision date: 7/25/2016 Revision: 38 Supersedes date: 7/20/2016 SAFETY DATA SHEET, PC 42 SEAWEED, Product name PC 42 SEAWEED Product number PC 42 SEAWEED, Recommended use of the chemical and restrictions on use Application ceramic glaze, Supplier American Art Clay Co Inc				
	5.	WHO FOOD ADDITIVES SERIES: 70; Prepared by the Seventy-ninth meeting of the Joint FAO/WHO Expert Committeeon Food Additives (JECFA)Safety evaluation of certain food additives, World Health Organization, Geneva, 2015				
	6.	Risk assessment report; Arsenic in foods(Chemicals and Contaminants) Food Safety commission. Japan (FSCJ), October 2013				
	7.	TOXICOLOGICAL PROFILE FORBARIUM AND BARIUM COMPOUNDS U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES Public Health ServiceAgency for Toxic Substances and Disease Registry, August 2007				
	8.	SCIENTIFIC OPINION EFSA Journal, ADOPTED: 14 March 2018, doi: 10.2903/j.efsa.2018.5238 Re-evaluation of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) as food additives				
	9.	Safety Data Sheet, Maxicrop Liquid Seaweed Ohrstrom's Maxi-crop Liquid Seaweed; Maxi-crop Liquid Seaweed, Maxi-crop Liquid Seaweed 0-0-1 Maxi-crop USA, Inc.				

4.1.3: Bio-chemicals

S.No.	Biostimulants	LD50 mg/kg	NOAEL/ LOAEL mg/kg/day ppm	Tox Profile		Global Regulations / Guidelines
				Symptoms	Effects	
	Biochemicals Polyethylene glycol, 8000; Polyethylene Glycol 2000 & 3400 D- Mannitol Dextran 70 Myo-inositol Tris (hydroxyl-methyl) amino methane L-Aspartic Acid; Sodium Chloride Sodium thiocyanate EDTA tetrasodium salt Cupric Sulfate Pentahydrate (2)	Acute Oral (Rat) (2) > 50000 13,500 10,700 10,000 5900 5000 3000 764 630- 1,260 300 Oral (rat) > 2000 Dermal (rabbit) > 2000 Inhalation LC50 (rat) > 20 mg/litre/4h. (3) 37% Formaldehyde oral-rat 800 (4)	Table 3-1 Levels of Significant Exposure to Benzene – Inhalation: 300 F/2200 F (decreased maternal body weight) (5) Table 3-2 Levels of Significant Exposure to Selenium – Oral Rat - 0.251 F (6)	Cough, shortness of breath, headache, nausea.; inhaled dust, mist: may cause respiratory irritation; causes severe skin burns and eye damage/irritation (1). Eye irritation may cause permanent damage; Ingestion May cause allergic reaction. (4)	Destructive to tissue of the mucuous membranes and upper respiratory tract. (1) Harmful if swallowed; inhaled; harmful to aquatic life with long lasting effects (1) Reproductive effects to women at high doses (2) Low order of acute toxicity (3).	No-observed-effect level (NOEL) and no-observed-adverse-effect level (NOAEL): use in animal health risk assessment (8) <i>Principles and Methods for the Risk Assessment of Chemicals in Food (2009)</i> , WHO Environmental Health Criteria, No. 240 have been adopted EFSA(7) & (9) Three step method by applying the weight-based classification to the NOEL, NOAEL and LOAEL based on the findings.(10)
References:						
1.	Trifluoro acetic acid, biochemical grade; Safety Data Sheet 2121302 = SYNQUEST Laboratories according to Federal Register / Vol. 77, No. 58 / Monday, March 26,					

	2012 / Rules and Regulations; Date of issue: 05/24/2017 Version: 1.0
2.	MATERIAL SAFETY DATA SHEET (MSDS)/SAFETY DATA SHEET (SDS) – ABAXIS; Biochemistry Panel Plus. 005-9025 Rev D DCO#: 50156 Effective: 05/21/15 Page 1 of 6 Product Name: Piccolo® Reagent Disc
3.	BIOCHEMICAL REAGENTS – BRS Trade name BIOCHEMICAL REAGENTS; Product No. BRS Date of issue – 10/02/2009.
4.	BBC Biochemical MATERIAL SAFETY DATA SHEET Section 1. Chemical Product and Company Information Common Name: 10% Neutral Buffered Formalin
5.	TOXICOLOGICAL PROFILE FOR BENZENE U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES Public Health Service Agency for Toxic Substances and Disease Registry August 2007
6.	TOXICOLOGICAL PROFILE FOR SELENIUM U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES Public Health Service Agency for Toxic Substances and Disease Registry September 2003
7.	European Food Safety Authority. (2017). Peer review of the pesticide risk assessment of the potential endocrine disrupting properties of glyphosate. EFSA Journal, 15, e4979. https://doi.org/10.2903/j.efsa.2017.4979
8.	Australian Govt; Australian Pesticides & Vet Meds Authority, 2019
9.	European Food Safety Authority. (2017). Quillaia extract/kg bw per day – NOAEL=400,750,1500
10.	Toxicol. Res.: Vol. 27, No. 3, pp. 133-135 (2011) Open Access http://dx.doi.org/10.5487/TR.2011.27.3.133 A New Way in Deciding NOAEL Based on the Findings from GLP-Toxicity Test Yeong-Chul Park ¹ and Myung-Haing Cho ² ¹ GLP Center, Catholic University of Daegu, Kumrak 5-Ro, Hayang-Up, Kyongsan-Si, Kyongbuk 712-702, Korea ² Laboratory of Toxicology, College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea

4.1.4. Antioxidants

S.No,	Biostimulants	LD50	NOAEL/LOAEL	Tox Profile		Global Regulations/Guidelines
				Symptoms	Effects	
	Antioxidants	Acute Oral >5000 Dermal >2000(2)	100 mg/kg body mass/day for poultry (EFSA, 2017) (7) approximately 400, 750, 1500 mg/kg bw (EFSA 2019) (9) LD50 was in excess of 2000 mg/kg, the NOAEL in excess of 61.9	Eye, skin, respiratory irritation(1)) May be harmful if swallowed (3) May cause an allergic skin reaction	Expected to have low acute oral, dermal & inhalation toxicity (1)	No-observed-effect level (NOEL) and no-observed-adverse-effect level (NOAEL); use in animal health risk assessment (4) <i>Principles and Methods for the Risk</i>

			mg/kg per day and the LOAEL in excess of 121 mg/kg (8) Protocol for standardized methodology to estimate the no-observed-adverse-effect-level (NOAEL) (5) Hormesis induced by physical or chemical agents in plants, with a modest maximum stimulatory response commonly less than twofold the control response occurring at a <100-fold distance from the no-observed-adverse-effect level (NOAEL) (6)			<i>Assessment of Chemicals in Food</i> (2009), WHO Environmental Health Criteria, No. 240 have been adopted EFSA(7) & (9)
		Fish LC50 (96 h) > 71 mg/l, (2)		Repeated ingestion of high doses, may cause damage to the heart in animals. Observed effects were reversible (2)		
References:						
1.	SAFETY DATA SHEET - MORRIS antioxidant: Oxide Inhibitor Compound/Petroleum-based oxide inhibitor					
2.	Material Safety Data Sheet: Antioxidant DLTDP/Irganoxps 800 - Chemical name: didodecyl 3,3'-thiodipropionate					
3.	MATERIAL SAFETY DATA SHEET; Antioxidant 702 Product Name 4,4,-METHYLENEBIS(2,6-DI-TERTProduct					

4.	Australian Govt; Australian Pesticides & Vet Meds Authority, 2019
5.	MethodsX 8 (2021) 101568; Protocol Article Estimating the no-observed-adverse-effect-level (NOAEL) of hormetic dose-response relationships in meta-data evaluations Evgenios Agathokleous a , *, Michael N Moore b , c , d , Edward J Calabrese e
6.	Opinion (Article in Press – Cell Press Reviews) Hormesis: A Compelling Platform for Sophisticated Plant Science Evgenios Agathokleous ,1,2,* Mitsutoshi Kitao,2 and Edward J. Calabrese3
7.	European Food Safety Authority. (2017). Peer review of the pesticide risk assessment of the potential endocrine disrupting properties of glyphosate. EFSA Journal, 15, e4979. https://doi.org/10.2903/j.efsa.2017.4979
8.	Cremer, D.R.; Rabeler, R.; Roberts, A.; Lynch, B. Safety evaluation of alpha-lipoic acid (ALA). Regul. Toxicol. Pharmacol. 2006, 46, 29–41. Above is cross ref from foll. Article Toxicological Profile of the Pain-Relieving Antioxidant Compound Thioctic Acid in Its Racemic and Enantiomeric Forms Elena Lucarini et al. Published: 14 August 2020
9.	European Food Safety Authority. (2017). Quillaia extract/kg bw per day – NOAEL=400,750,1500

4.1.5: Microorganisms

S.No.	Biostimulants	LD50mg/kg	NOAEL /LOAEL mg/kg/day ppm	Tox Profile		Global Regulation/ Guidelines
				Symptoms	Effects	
	Microorganisms Tannic acid	Fish LC50 (96hr.) (mg/l): 100 (6)		Ingestion Maybe harmful if swallowed. Inhalation Prolonged inhalation may lead to respiratory tract irritation. Eye Prolonged or repeated exposure may cause Skin Prolonged or repeated exposure may cause irritation, redness, and inflammation. Effects of overexposure Long term	Eyes: Possible arduency and redness. Skin: Non-irritating. Ingestion : Non-toxic natural bacterial cells. Do not ingest. Inhalation: Non-irritating. (3) Repeated exposure to high concentr	GUIDELINES / DATA REQUIREMENTS FOR REGISTRATION OF ENTOMOPATHOGENIC FUNGI UNDER SECTION 9(3B) and 9(3) OF THE INSECTICIDE ACT, 1968 (w.e.f. 1st January, 2011) (1) Regulatory authorities generally require sufficient information on a microbial pesticide to characterize it, to assess its potential risks to people and

				exposure may cause mechanical irritation of the eyes as well as skin and respiratory tract irritation. Existing conditions aggravated by exposure May provoke asthmatic response in persons with asthma who are sensitive to airway irritants. (4) Signs and Symptoms of Exposure (progressive): ● Inhalation: Sneezing, mucous flow, coughing ● Skin/eyes: Skin coloring, eyes irritation, tearing ● Aqueous solution discolors skin, but no permanent adverse effects. No toxic effect known from dust inhalation or ingestion. (6)	ations of microbial proteins can cause allergic sensitization.(5)	to the environment, and to confirm its effectiveness for pest control. Unlike chemical pesticides, microbial pest control agents may infect or cause disease in other living organisms.(2) Repeated exposure to high concentrations of microbial proteins can cause allergic sensitization. (5)
--	--	--	--	--	--	---

References:

1.	CIB&RC, Min of Agr & Farmer's Welfare GUIDELINES / DATA REQUIREMENTS FOR REGISTRATION OF ENTOMOPATHOGENIC FUNGI UNDER SECTION 9(3B) and 9(3) OF THE INSECTICIDE ACT, 1968 (w.e.f. 1st January, 2011)
2.	OECD Guidance for Industry Data Submissions for Microbial Pest Control Products and their Microbial Pest Control Agents (Dossier Guidance for Microbials) February 2004 -- OECD Environment Directorate -
3.	SAFETY DATA SHEET

	AGRO GOLD SD Product Name: Agro Gold SD Product Use: Biological amendment containing soil enhancing agriculture bacteria. Supplier: AGRO RESEARCH INTERNATIONAL Inc.
4.	Safety Data Sheet – New edge Microbials Product Name: EasyRhizTM Issue Date: October 2020 Version: 3.0 Page: Page 1 of 6 1. Identification of The Material and Supplier Product Name EasyRhizTM Legume Inoculant Other Names Freeze dried legume inoculant
5.	Indigo® Biological Fungicide 97464WP Revision Date: 23OCT2018 SAFETY DATA SHEET
6.	NATURAL DYE MATERIAL SAFETY DATA SHEET SECTION 1--COMPANY IDENTITY BOTANICAL COLORS, LLC 10550 PHINNEY AVE N SEATTLE, WA 98133 USA

4.1.6: Protein hydrolases & amino acids

S.No,	Biostimulants	LD50mg/kg	NOAEL /LOAEL mg/kg/day ppm	Tox Profile		Global Regulation/ Guidelines
				Symptoms	Effects	
	Protein hydrolases & amino acids H314(2) H318(2) Amino acids (3)	0.5–3.5 mg/kg body weight (bw).(4)	6 months -6 year Resp 109wk 7d/wk 3-4.5hr/d (Table 2.1) Chronic exposure LOAEL values Above (Table 2.2) Acute exposure LOAEL values below 150 M 40F 10F (1)	Causes skin irritation. Causes serious eye irritation (3)	Causes severe skin burns and eye damage. Causes serious eye damage.(2)	
References:						
1.	TOXICOLOGICAL PROFILE FOR POLYCYCLIC AROMATIC HYDROCARBONS U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES					

	Public Health Service Agency for Toxic Substances and Disease Registry August 1995
2.	Safety Data Sheet BIOSynth (Carbosynth) Release Revision Date of issue 1.0 11/25/2020 04/22/2022 page 1 of 6 According to Appendix D, OSHA Hazard Communication Standard 29 CFR §1910.1200 1 Identification of the substance 1.1 Product identifier Identification of the substance (R)-(-)-2-Phenylglycinol
3.	WATERS CORPORATION Product: Amino Acid Standards: WAT088122, WAT010948, WAT010949 MSDS #: 735000122 Product Use: For laboratory use only. Date: Rev 3, May 2, 2013
4.	EFSA Journal SCIENTIFIC OPINION ADOPTED: 1 March 2016 doi: 10.2903/j.efsa.2016.4424 Acute health risks related to the presence of cyanogenicglycosides in raw apricot kernels and products derived from raw apricot kernels. EFSA Panel on Contaminants in the Food Chain (CONTAM)

4.1.7: Vitamins

S.No,	Biostimulants	LD50mg/kg	NOAEL /LOAEL mg/kg/day ppm	Tox Profile		Global Regulation/ Guidelines
				Symptoms	Effects	
	Vitamins Vitamin A (preformed) (5) Vitamin D(5) Vitamin Ef,g(5) Vitamin K(5) Vitamin C(5) Niacin(5) Vitamin B6(5) Thiamin (vitamin B1)(5) Riboflavin (vitamin B2)(5) Folate (5)	High doses (up to the safe upper limit—2,000 milligrams a day) of vitamin C are usually not toxic to healthy adults. (1) Serum 25-hydroxyvitamin D [25(OH)D] concentrations higher than 150 ng/ml (375 nmol/l) are the hallmark of VDT due to vitamin D overdosing. (2) Potential chronic toxicity would result from administration of doses above 4,000 IU/day for extended periods, possibly for years, that cause serum 25(OH)D concentrations in the 50–	NOAEL 3,000 mcg LOAEL 14,000 mcg, NOAEL 60 mcg LOAEL 500 mg/kg NOAEL 80 mcg NOAEL 2000 mg LOAEL 3000 mg LOAEL = 50 mg NOAEL = 200 mg NOAEL 1.5 mg NOAEL 1.7 mg LOAEL = 5 mg (5)	higher doses cause nausea or diarrhea and interfere with the balance of antioxidant activity in the body. (1) The clinical manifestations of VDT are varied but are related primarily to hypercalcemia (3, 5). Symptoms of VDT may be similar to those of other hypercalcemic states and include neuropsychiatric manifestations, such as difficulty in concentration, confusion, apathy, drowsiness, depression, psychosis, and in extreme cases, a stupor and coma. The gastrointestinal symptoms of VDT include recurrent vomiting, abdominal pain, polydipsia, anorexia, constipation,	Exogenous VDT is usually caused by the inadvertent or improper intake of extremely high doses of pharmacological preparations of vitamin D and is associated with hypercalcemia and in hypervitaminosis D, the concentrations of vitamin D metabolites (2) Toxic if swallowed. Toxic in contact with skin. Fatal if inhaled. Causes damage to organs through prolonged or repeated exposure.(4)	

	150 ng/ml (125–375 nmol/l) (2) H301: H311: H330: H372: Oral TDLO (infant): 39 mg/kg/34W (intermittent); Oral LD50 (rat): 42 mg/kg; Oral LD50 (mouse): 42500 µg/kg; Intraperitoneal LD50 (mouse): 136 mg/kg; Subcutaneous TDLO (rat): 90 mg/kg; range (4)		peptic ulcers, and pancreatitis. The cardiovascular manifestations of VDT include hypertension, shortened QT interval, ST segment elevation, and bradyarrhythmias with first-degree heart block on the electrocardiogram. The renal symptoms include hypercalciuria as the earliest sign, polyuria, polydipsia, dehydration, nephrocalcinosis, and renal failure. Other symptoms of VDT caused by hypercalcemia include band keratopathy, hearing loss, and painful periarticular calcinosis (2) Vitamin E toxicity can cause major bleeding events. These can be serious, including the potential for intracranial hemorrhage. (2) Symptoms are not noticed until levels beyond 1000 mg are ingested daily. However, drug-	
--	---	--	--	--

				drug interactions have been reported in patients ingesting more than 300 mg of vitamin E daily. (3) May be irritating to the mucous membranes and upper respiratory tract. May cause eye, skin, or respiratory system irritation. Toxic if swallowed or in contact with skin.(4)		
	Vitamin D3 Riboflavin 5'-(sodium hydrogen phosphate) Pyridoxine Hydrochloride: Cyanocobalamin (6)	Acute oral toxicity: LD50 (Rat): > 20,000 mg/kg LD50 (Rat): 4,000 mg/kg LD50 (Rat): > 5,000 mg/kg (6)				
References:						
1.	(Vitamin C Toxicity)					
2.	REVIEW article Front. Endocrinol., 20 September 2018 https://doi.org/10.3389/fendo.2018.00550 Vitamin D Toxicity–A Clinical Perspective Ewa Marcinowska-Suchowierska1*, Małgorzata Kupisz-Urbańska1, Jacek Łukaszkiwicz2, Paweł Płudowski3 and Glenville Jones4					
3.	StatPearls [Internet].					

	Show details Search term Vitamin E Toxicity Kristen N. Owen; Olga Dewald. Author Information Last Update: November 14, 2021.
4.	SAFETY DATA SHEET – Cayman Chemicals Supersedes Revision: 11/12/2014 according to Regulation (EC) No. 1907/2006 as amended by (EC) No. 2015/830 and US OSHA HCS 2015 1.1 Product Code: 11792 Section 1. Identification of the Substance/Mixture and of the Company/Undertaking Product Name: Vitamin D3
5.	Upper Safe Levels of Intake for Adults: Vitamins, Macrominerals, and Trace Minerals By Judy A. Driskell, Professor of Nutritional Science and Dietetics
6.	SAFETY DATA SHEET - MERCK Multivitamin Aqueous Formulation Version 3.0 Revision Date: 10/10/2020 SDS Number: 4248872-00005 Date of last issue: 03/23/2020 Date of first issue: 05/06/2019

4.1.8: Humic acid and Fulvic acid

S.No,	Biostimulants	LD50mg/kg	NOAEL /LOAEL mg/kg/day ppm	Tox Profile		Global Regulation/ Guidelines
				Symptoms	Effects	
1.	Humic and Fulvic acid H313 H315 H319 & H320 H335 H302	Acute Oral (Rat) 273 mg/kg (Potassium Hydroxide)(4)	No general or organ toxicity was observed in Wistar rats following 90 days of continuous exposure, and a no observed adverse effect level (NOAEL) was determined at 2000 mg/kg bw/day, the highest tested dose.(2) (EFSA) evaluated an	May be harmful in contact with skin (4) Causes skin irritation. Causes serious eye irritation. May cause respiratory irritation.(	Skin irritation (Category 2), Eye irritation (Category 2), Specific target organ toxicity - single exposure (Category 3), (1)	The public comment period will be open for 30 days in docket EPA-HQ-OPP-2018-0258 at www.regulations.gov . After carefully considering the comments received, EPA

			unpublished subchronic toxicity study in rats of a mixture of humic and fulvic acids with added minerals and determined a no observed adverse effect level (NOAEL) of 50 mg/kg bw/day due to body and organ weight decreases at higher doses that could not be laid to rest due to the absence of histological evaluations (3). A NOEAL of 15 mg/kg bw/day of potassium humate was determined in an unpublished chronic study in dogs due to the occurrence of vomiting and watery feces at 50 mg/kg bw/day and mild heart and liver lesions at 150 mg/kg bw/day.(2)	1) Harmful if swallowed May be irritating to mouth, throat, and stomach. (4)	(5) Hazardous to the aquatic environment, acute hazard (5)	anticipates finalizing this guidance in January 2021.
--	--	--	--	---	--	---

References:

2.	cdhfinechemicals.com HUMIC ACID; CAS NO 1415-93-6 MATERIAL SAFETY DATA SHEET, SDS/MSDS
2.	Murbach TS, Glávits R, Endres JR, Clewell AE, Hirka G, Vértesi A, Béres E, PasicsSzakonyiné I. A toxicological evaluation of a fulvic and humic acids preparation. Toxicol Rep. 2020 Sep 14;7:1242-1254. doi: 10.1016/j.toxrep.2020.08.030.
3.	EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS),Chromium(III)-, iron(II)- and selenium-humic acid/fulvic acid chelate andsupplementedhumifulvate added for nutritional purposes to food supplements,Efsa J. 1147 (2009) 1–12036.
4.	Safety Data Sheet (SDS) - actagro

	OSHA HazCom Standard 29 CFR 1910.1200(g) and GHS Rev 03. Issue date 05/05/2015 Reviewed on 04/05/2018 40.3.3; * 1 Identification;· Product identifier;· Trade name: Humic Acid 10%
5.	SAFETY DATA SHEET – Plant Food Company Inc. Humic Acid 70, Granular Humic and fulvic acid derived from Leonardite
6.	EPA Seeking Comments on Updated Plant Biostimulants Guidance November 24, 2020; Contact Information EPA Press Office (press@epa.gov)

Chapter 5: Methods for quantification of biostimulants

5.1: Method for quantification of humic content in a product

- International Organization for Standardization Geneva, Switzerland **ISO 19822:2018** method for Determination of humic and hydrophobic fulvic acids concentrations in biostimulants.

[Note 1: The concerned stakeholders may purchase the detailed method from International Organization for Standardization Geneva, Switzerland]

[Note 2: CFQCTI, Faridabad or Division of Agricultural Chemicals, IARI may be made as referral laboratory for determination of humic content in biostimulants]

5.2: Biostimulants Characterization

- Botanical extracts including seaweed extracts and cell-free microbial extracts consists of large number of different molecules. These can be characterized by gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry (LC-MS) techniques. In GC-MS method is suitable for analysis of volatile metabolites and metabolites that can be converted into volatile after derivatization, examples, fatty acids, amino acids, sugars, and organic acids. The LC-MS method separates compounds in the liquid phase. Metabolites such as amino acids, sugars, organic acids, flavonoids, alkaloids, and phenylpropanoids, etc, can be detected in this platform. LC-MS/MS and GC-MS/MS can detect most metabolites and hormones.
- Referral laboratories with LC-MS/MS and/or GC-MS/MS can be considered for characterization of all classes of biostimulants (Jorge et al. 2016; Patel et al. 2021; Ma and Qi 2021). Alternatively, data from laboratories with National Accreditation Board for Testing and Calibration Laboratories (NABL) accreditation & Good Laboratory Practice (GLP) certification can be used for analysing the biostimulants.

- **References:**

Jorge TF, Mata AT, António C. 2016. Mass spectrometry as a quantitative tool in plant metabolomics. *Phil. Trans. R. Soc.* A374: 20150370. <http://dx.doi.org/10.1098/rsta.2015.0370>.

Patel MK, Pandey S, Kumar M, Haque MI, Pal S, Yadav NS. 2021. Plants Metabolome Study: Emerging Tools and Techniques. *Plants* (Basel). 10(11):2409. doi: 10.3390/plants10112409.

Ma A, Qi X. 2021. Mining plant metabolomes: Methods, applications, and perspectives. *Plant Commun.* 2(5):100238. doi: 10.1016/j.xplc.2021.100238.

5.3: Seaweed Extracts

- Seaweed extracts are prepared from Red (*Rhodophyceae*), brown (*Phaeophyceae*) and green (*Chlorophyceae*) algae grown in marine and coastal ecosystems.
- Some the common seaweed species used for preparation of seaweed extracts are listed in Table 5.3.1.

Table 5.3.1: Common seaweed species used for biostimulant preparation

Phaeophyceae	Rhodophyceae	Chlorophyceae
<i>Ascophyllum nodosum</i>	<i>Macrocystis pyrifera</i>	<i>Ulvalactuca</i>
<i>Ecklonia maxima</i>	<i>Porphyra perforate</i>	<i>Enteromorpha prolifera</i>
<i>Durvillea antarctica</i>	<i>Nereocystis</i> spp.	<i>Caulerpa paspaloides</i>
<i>Durvillea protatorum</i>	<i>Cyanidium caldarium</i>	<i>Ulva armoricana</i>
<i>Fucus vesiculosus</i>	<i>Gelidium serrulatum</i>	<i>Codium Liyengarii</i>
<i>Sargassum</i> spp.	<i>Acanthophora spicifera</i>	<i>Codium tomentosum</i>
<i>Hydroclathrus</i> spp.	<i>Kappaphycus Alvarezii</i>	<i>Caulerpa sertularioides</i>
<i>Ralfsia</i> spp.	<i>Gracilaria edulis</i>	
<i>Laminaria digitata</i>	<i>Gracilaria dura</i>	
<i>Cystoseira myriophylloides</i>	<i>Laurencia johnstonii</i>	
<i>Fucus spiralis</i>		
<i>Padina pavonica</i>		
<i>Fucus gardneri</i>		
<i>Durvillea antarctica</i>		

- For seaweed extract preparation, both physical (heat, pressure and microwaves) and chemical (solvents, acids, and alkali) methods are employed. Seaweed extracts consist of a wide group of biochemicals such as polysaccharides, plant hormones, fatty acids, sterols, carotenoids, oxylipins, minerals, peptides, amino acids and proteins, lipids, polyphenolics, phlorotannins, etc. Of these about 60% are polysaccharides, 15% are proteins, 2% each of lipids, minerals and plant hormones, and rest are other compounds.
- This category of biostimulant can be characterised by using LC-MS/MS or GC-MS/MS. Some of the important molecules may be quantified to assess the quality and characteristics of seaweed as described in Table 5.3.2.

Table 5.3.2: Methods for quantification of molecules in seaweed extracts

Chemicals to be quantified	Method of determination	BIS specification	Remarks
Agar	Matsushashi and Hayashi 1972; Villanueva et al. 2011; Meena et al. 2011	IS 6850: 1973 and IS 5707: 1996	Red algae (<i>Rhodophyceae</i>)
Carrageenan	Craigie and Leigh, 1978; Eswaran et al. 2002	IS 16147: 2014	Red algae (<i>Rhodophyceae</i>)
Alginic acid and alginates	Chee et al. 2011; Prasad et al. 2017	IS 5191: 1993	Brown Algae (<i>Phaeophyceae</i>)
Fucoidan	Hahn et al. 2012		Brown Algae

			(<i>Phaeophyceae</i>)
3,6-anhydrogalactose	Yaphe and Arsenault, 1965		All species
Phyto hormones	Prasad et al. 2010; Das and Prasad 2015		All species
Total Sugars	Dubois et al. 1956		All species
Total Nitrogen	Kjeldahl method (AOAC, 1999a)		All species
Minerals	Inductively Coupled Plasma Optical Emission spectroscopy (ICP-OES) Method		All species; All minerals other than C, H, O, N and the halogens can be determined.
Different carbohydrates	FTIR		
Total phenolics	Fu et al. 2016		

• **References:**

1. Chee SY, Wong PK, Wong CL. 2011. Extraction and characterisation of alginate from brown seaweeds (Fucales, Phaeophyceae) collected from Port Dickson, Peninsular Malaysia. *Journal of Applied Phycology* 23: 191–196
2. Craigie JS, Leigh C. 1978. Carrageenans and agars. **In** J. A. Hellebust, & J. S. Craigie (Eds.), *Handbook of phycological methods: Physiological and biochemical methods* (pp. 109–131). Cambridge: Cambridge University Press.
3. Das AK, Prasad K. 2015. Extraction of plant growth regulators present in *Kappaphycus alvarezii* sap by imidazolium based ionic liquids: Detection and quantification by HPLC–DAD technique. *Analytical Methods* 7: 9064–9067.
4. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry* 28: 350-356.
5. Eswaran K, Ghosh PK, Siddhanta AK, Patolia S, Periyasamy C, Mehta AS, Mody KH, Ramavat BK, Prasad K, Rajyaguru MR, Kulandaivel S, Reddy CRK, Pandya JB, Tewari A. 2002. *Integrated method for production of carrageenan and liquid fertilizer from fresh seaweeds*. US Patent 6,893,479.
6. Fu CWF, Ho CW, Yong WTL, Abas F, Tan TB, Tan CP. 2016. Extraction of phenolic antioxidants from four selected seaweeds obtained from Sabah. *International Food Research Journal* 23(6): 2363-2369
7. Hahn T, Lang S, Ulber R, Muffler K. 2012. Novel procedures for the extraction of fucoidan from brown algae. *Process Biochemistry* 47: 1691-1698.
8. Matsushashi T, Hayashi K. 1972. Agar processed from *Gracilariopsis lemaneiformis* of Florida. *Agricultural and Biological Chemistry*, 36(9): 1543-1552.
9. Meena R, Prasad K, Ganesan M, Siddhanta AK. 2011. Preparation of superior quality products from Indian agarophytes. *Journal of Applied Phycology* 23: 183-189.
10. Prasad K, Das AK, Oza MD, Brahmabhatt H, Siddhanta AK, Meena R, Eswaran K, Rajyaguru MR, Ghosh PK. 2010. Detection and quantification of some plant growth regulators in a seaweed-based foliar spray employing a mass

spectrometric technique sans chromatographic separation. *Journal of Agricultural and Food Chemistry* 58 (8): 4594–4601.

11. Prasad K, Maiti P, Mukesh C, Ghara KK, Meena R, Ghosh SC. 2017. A zero liquid discharge process for the production of alginic acid and its derivatives from *alginophytes*. Indian Patent Application No. 201711025753 dated 20-07-2017.
12. Villanueva RD, Sousa AMM, Gonçalves MP, Nilsson M, Hilliou L. 2010. Production and properties of agar from the invasive marine alga, *Gracilariavermiculophylla* (Gracilariales, Rhodophyta). *Journal of Applied Phycology* 22: 211–220
13. Yaphe W, Arsenault GP. 1965. Improved resorcinol reagent for the determination of fructose, and of 3,6-anhydrogalactose in polysaccharides. *Analytical Biochemistry* 13: 143-148.

5.4: Protein Hydrolysates and Free Amino Acids

- Protein hydrolysates contain mainly peptides and free amino acid.

Sources: legume seeds, alfa-alfa biomass, soymeal, fish by-products, collagen from leather by-products

Quantification of different amino acids, total protein content and total nitrogen content may be carried out to quantify and characterize biostimulants belonging to Protein hydrolysates and free amino acids.

- **Aminoacid composition analysis:**

UltraHighPerformance Liquid Chromatography (UHPLC/UPLC or HPLC) (Rutherford and Gilani 2009), and tandem mass spectrometry (LC–MS/MS) (Dahl-Lassen et al. 2018)

- **Total Nitrogen estimation:**

Kjeldahl Method (AOAC, 1999a); Dumas (AOAC, 1999b), and combustion (AOAC, 1999c)

- **Soluble protein estimation:**

Bradford's method (Bradford 1976; Stoscheck 1990); Lowry's method (Lowry et al. 1951)

- **References**

1. AOAC, 1999a. Official Methods of Analysis Method 988.05. Ch. 4, p. 13 AOAC International, Gaithersburg, Md.
2. AOAC, 1999b. Official Method of Analysis Method 968.06. Ch. 4, p. 13 AOAC International, Gaithersburg, Md.
3. AOAC, 1999c. Official Method 990.03. Ch. 4, p. 18 AOAC International, Gaithersburg, Md.
4. Bradford, MM. 1976. A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248-254.

5. Dahl-Lassen R, van Hecke J, Jørgensen H, Bukh C, Andersen B, Schjoerring JK. 2018. High-throughput analysis of amino acids in plant materials by single quadrupole mass spectrometry. *Plant Methods* 14:8.
6. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *J BiolChem* 193: 265–275
7. Rutherford SM, Gilani GS. 2009. Amino acid analysis. *CurrProtoc Protein Sci.* 58:11.19.11–37.
8. Stoscheck, CM. 1990. Quantitation of Protein. ***Methods in Enzymology* 182: 50-69.**

5.5: Humic and Fulvic Acids and their Derivatives

- Humic substances (HS) are decomposition products of plant and animal matter from chemical and biological transformations by microbial metabolism. HS may regulate soil fertility due to their ability to retain water and nutrients, to improve soilcation exchange capacity (CEC), to increase nutrient availability and to generate aeratedsoil structure.
- HS can be grouped into humic acids (HA, soluble at alkalinepH and insoluble at acidic pH), fulvic acids (FA, which are soluble both at alkaline and acidic pH) and humins (insoluble).
- **Quantification Method:**
International Organization for Standardization Geneva, Switzerland **ISO 19822:2018**method.

5.6: Vitamins

- **Vitamin C:**AOAC Official Method 967.21, Ascorbic Acid in Vitamin Preparations and Juices, 2,6-Dichloroindophenol Titrimetric Method, AOAC Official Methods of Analysis 45.1.14 (AOAC Method 967.21) has been recommended for the analysis of L-AA in beverages and juices for the purpose of nutrition labelling; The AOAC Official Method 985.33, Vitamin C (ReducedAA) in Ready-to-Feed Milk-Based Infant Formula);AOAC SMPR 2021.XXX; Version 3; February 25, 2021Standard Method Performance Requirements for Vitamin C in Infant Formula and 4 Adult/Paediatric Nutritional Formula.
- For all vitamins specific analytical methods are available.

5.7: Common seaweeds used for preparation of plant bio stimulants and active polysaccharide content

Table 5.7.1: Class and name of seaweed along with Polysaccharides obtained from them.

Class	Name of the seaweed	Polysaccharide
<i>Phaeophyceae</i>	<i>Ascophyllumnodosum</i>	Alginic acid/alginate, Fucoidan
	<i>Durvilleaprotatorum</i>	Alginic acid/alginate, Fucoidan, Laminarin
	<i>Durvilleaantarctica</i>	Alginic acid/alginate, Fucoidan, Laminarin
	<i>Ecklonia maxima</i>	Alginic acid/alginate, Fucoidan, Laminarin
	<i>Fucusvesiculosus</i>	Sulphated polysaccharides with mannose, galactose, glucose, or xylose

	<i>Sargassum sp.</i>	Alginic acid/alginate, Fucoidan
	<i>Hydroclathrus spp.</i>	alginic acid, fucoidan, and laminarin
	<i>Laminariadigitata</i>	Alginic acid/alginate, Fucoidan, Laminarin
	<i>Macrocystispyrifer</i>	Alginic acid/alginate, Fucoidan, Laminarin
	<i>Nereocystis spp.</i>	Alginic acid/alginate, Fucoidan
	<i>Cystoseira sp.</i>	Alginic acid/alginate
<i>Rhodophyceae</i>	<i>Acanthoporphyraspicifera</i>	Lambda-carrageenan
	<i>Cyanidium caldarium</i>	Glycogen type glucan
	<i>Gelidium serrulatum</i>	Agar
	<i>Gracilaria edulis</i>	Agar
	<i>Gracilaria dura</i>	Agar
	<i>Kappaphycus alvarezii</i>	Kappa carrageenan
	<i>Laurencia johnstonii</i>	Galactan
	<i>Porphyra perforate</i>	Porphyrin, complex galactan
<i>Chlorophyceae</i>	<i>Caulerpa paspaloides</i>	Glucan, Glycan
	<i>Caulerpa sertularioides</i>	Glucan, Glycan
	<i>Codium Lyngbyi</i>	Galactan, Arabinan, Mannans
	<i>Codium tomentosum</i>	Galactan, Arabinan, Mannans
	<i>Enteromorpha prolifera</i>	Polysaccharide composed of monosaccharides: rhamnose, glucuronic acid, and xylose
	<i>Ulva armoricana</i>	Ulvan
	<i>Ulva lactuca</i>	Ulvan

5.8: Wet methods for extraction of seaweed constituents and their estimation

- Method of extraction of polysaccharides present in red, brown and green seaweeds are as below:

5.8.1: Agar:

- Accurately weighted sample # to be soaked in 200 mL tap water for 1 h at room temperature and then will be treated with 200 mL of 8% w/v aqueous NaOH solutions at 90°C in a water-bath for 2 h. After the alkali treatment, excess alkali is to be removed by water washing until the washing showed pH in the range of 7–8. The cooked seaweed will then homogenized in a grinder mixture, filtered through a celite bed under vacuum to obtain the clear extract, which will be finally precipitated in isopropyl alcohol (1: 3 v/v). The final product will be obtained after vacuum dry [1-3].

Table 5.8.1.1: Parameters (Confirmatory tests) to be analysed for AGAR and the range of the values (As per IS : 6850 : 1973)

Parameter	Range	Range as per IS 6850 : 1973 and IS 5707 : 1996
Appearance	--	White to pale yellow in colour
Gel strength for 1.5% gel in water	150 to 2000 g/cm ²	NA
Gelling temperature	34-41 °C	NA
Melting temperature	90-100 °C	NA
Moisture content (%)	-	<20
Total Ash content (%)	-	<6.5
Gelatin content	-	NIL
Acid insoluble ash (%)	-	<1.0
Insoluble matter (%)	-	<1.0

Arsenic (as As) (mg/kg)	-	<3.0
Lead (as Pb) (mg/kg)	-	<10.0
pH (1.5% aqueous solution)	-	6-8

[#] for liquid, Total dissolved solid (TDS) need to be used

NA : Not applicable (Not mentioned in Indian standards)

Table 5.8.1.2: Some more characteristics (Confirmatory tests) of agar as per IS 6850 : 1973 and IS 5707 : 1996 (Indian Standard SPECIFICATION FOR AGAR, MICROBIOLOGICAL/FOOD GRADE)

Parameters	Requirement
Solubility and gelation	It shall form a clear solution just below 100°C when heated with excess of water. On cooling, a concentration of not more than 2 %shall solidify to form a stable gel at not more than 40°C. This gel shall not liquefy below 85°C.
	When autoclaved at 121°C for 30 minutes and then cooled, it shall form a stable gel.
	After autoclaving twice at 121°C for 30 minutes, a 2 %aqueous solution of agar shall maintain a stable gel on cooling.
	The agar shall have clarity when dissolved in media and shall be reasonably free from any suspended material.
Growth of bacterial colonies (Microbiological grade agar)	It shall not inhibit the growth of micro-organisms (e.g., <i>Escherichia coli</i>) when it is incorporated in a medium.
Total plate count (Food grade agar)	<5000 CFU
Coliforms/10 g (Food grade agar)	Absent
Salmonella /10 g (Food grade agar)	Absent
Yeasts and Moulds / g (Food grade agar)	<500

5.8.2: Carrageenan:

- Accurately weighted sample [#]to be soaked for 2 h in 0.5% calcium hydroxide solution (1:30 w/v). Further water equivalent to 10 times of the solid weight of sample will be added and will be autoclaved for 1.5 h at 107 °C. The hot extract obtained after the autoclave process will be ground in a blender and centrifuged. The pH of the supernatant to be maintained at 7.9. This final solution will be slowly added into isopropyl alcohol (1: 3 v/v) with constant stirring (1:3, v/v). The resulting carrageenan precipitates will be dried under vacuum [4,5].

Table 5.8.2.1: Parameters (Confirmatory tests) to be analysed for Carrageenan and the range of the values (As per IS 16147: 2014)

Parameter	Range	Range as per IS 16147: 2014
Gel strength for 1.0% gel in 1% KCl	150 to 800 g/cm ²	NA
Gelling temperature	30-40 °C	NA

Melting temperature	80-90 °C	NA
Loss on drying, %by mass	-	< 12
pH	-	8-11
Viscosity (cP, at 75°C of 1.5% solution)	-	> 5
Sulphate (% as SO ₄ ⁻²)	-	15-40
Total ash (% on dry basis)	-	15-40
Acid insoluble ash (% on dry basis)	-	< 1
Acid insoluble matter (% on dry basis)	-	< 2
Residual solvents, %by mass of ethanol, isopropanol, or methanol, singly or in combination (% by mass)	-	< 1
Arsenic (as As), mg/kg,	-	< 3
Lead (as Pb), mg/kg,	-	< 5
Cadmium (as Cd), mg/kg	-	< 1.5
Mercury (as Hg), mg/kg	-	< 1
Total (aerobic) plate count, cfu/g	-	< 5000
<i>Salmonella</i> spp	-	Absent
<i>E. coli</i> /g	-	Absent

[#] for liquid, Total dissolved solid (TDS) need to be used

NA : Not applicable (Not mentioned in Indian standards)

Table 5.8.2.2: Some more characteristics (Confirmatory tests) of Carrageenan as per IS 16147: 2014 (Indian Standard CARRAGEENAN, FOOD GRADE — SPECIFICATION)

Parameters	Requirement
Solubility and gelation	The material shall be insoluble in ethanol and shall be soluble in water at a temperature of about 80 °C forming a viscous clear or slightly opalescent solution that flows readily. The material shall disperse in water more readily if first moistened with alcohol, glycerol, or a saturated solution of glucose or sucrose in water
	Add 4 g of sample to 200 ml of water, and heat the mixture in a water bath at 80°C, with constant stirring, until dissolved. Replace any water lost by evaporation, and allow the solution to cool to room temperature. It becomes viscous and may form a gel. To 50 ml of the solution or gel add 200 mg of potassium chloride, then reheat, mix well, and cool. A short-textured ('brittle') gel indicates a carrageenan of a predominantly kappa type, and a compliant ('elastic') gel indicates a predominantly iota type. If the solution does not gel, the carrageenan is of a predominantly lambda type.

5.8.3: Alginic acid and alginates:

- 0.4N H₂SO₄ solution to be added to accurately weighed sample [#] (1:8 w/v) and will be soaked for 24 h. Acidic filtrate obtained after centrifugation of the mixture will be discarded and residue to be washed with water to remove acid impurities followed by the addition of 5% w/v Na₂CO₃ (1:10 w/v) solution and will be kept in water-bath at 80 °C for 4 h. 1 N HCl will be added to the solution to bring the pH to 2.2 which will precipitate alginic acid. Alginic acid thus obtained will be solubilized in 5% NaOH solution and will be precipitated in isopropyl alcohol (1: 3 v/v) to get sodium alginate [6,7].

[#] for liquid, Total dissolved solid (TDS) need to be used

Table 5.8.3.1: Parameters (Confirmatory tests) to be analysed for Sodium alginate and the range of the values (As per **IS 5191: 1993**)

Parameter	Range	Range as per IS 5191: 1993
Purity (% by mass)		> 91
Loss on drying, %by mass	-	< 15
pH	-	8-11
Viscosity (cP, at 75°C of 1.0% solution)	-	> 30
Total ash (% on dry basis)	-	18-27
Acid insoluble ash (% on dry basis)	-	< 0.5
Matter insoluble in water (% on dry basis)	-	< 1
Arsenic (as As), mg/kg,	-	< 3
Lead (as Pb), mg/kg,	-	< 10
Cadmium (as Cd), mg/kg	-	< 1.5
Heavy metals (as Pb) mg/kg	-	< 40
<i>Salmonella</i> spp/10 g	-	Absent
<i>E. coli</i> /g	-	Absent

Table 5.8.3.2: Some more characteristics (Confirmatory tests) of Sodium alginate as per IS 5191: 1993 (*Indian Standard SODIUMALGINATE,FOODGRADE- SPECIFICATION*)

Parameter	Specification
Description	A white to yellow-white, fibrous powder, odourless.
Solubility	Slowly soluble forming a viscous solution in water; insoluble in ethanol, ether and chloroform.
Identification	(i) To a 0.5 % solution of the sample in sodium hydroxide add one-fifth of its volume of a 2.5 % aqueous solution of calcium chloride. A voluminous, gelatinous precipitate forms. This test distinguishes sodium alginate from arabic gum, carboxymethyl cellulose, Carboxymethyl starch, carrageenan, gelatine,Ghatti gum, karaya gum, locust bean gum, methyl cellulose, pectin and tragacanth.
	(ii) To a 0.5% solution of the sample in sodium hydroxide solution add one-half of its volume of a saturated solution of ammonium sulphate. No precipitate is forms. This test distinguishes sodium alginate from agar, carboxymethyl cellulose, carrageenan, de-esterified pectin, gelatine, locust bean gum, methyl cellulose and starch.

• **Some more characteristics (Confirmatory tests) of Alginic acid as per IS 5191: 1993**

- Take a quantity of material equivalent to 5 mg of alginic acid in a test-tube. Add 5 ml of water, 1 ml of a freshly prepared 1 in 100 solution of naphthoresorcinol in ethanol and 5 ml of concentrated hydrochloric acid. Heat the mixture to boiling. Boil gently for about 3 minutes and then cool to about 15°C. Transfer the contents of the test-tube to a 30-ml separator with the aid of 5 ml of water and extract with 15 ml of isopropyl ether. Perform the blank using the same quantities of the same reagents by the same procedure omitting the sample. The isopropyl ether extract from the material shall exhibit a deeper purplish hue than that from the blank.

- Moisten 1-5 mg of the sample with water, and add 1 ml of acid ferric sulphate solution. Within 5 minutes, a cherry-red colour develops that finally becomes deep purple.
- Dissolve the sulphated ash of the sample in dilute acetic acid solution and filter. Add to the filtrate uranyl zinc acetate solution. A yellow, crystalline precipitate is formed within a few minutes.

5.8.4: Fucoidan:

- The milled sample will be mixed with 95% ethanol in the ratio (1:2) and shaken well for 1 hour to remove pigments, proteins and lipids and then centrifuged for 10 minutes. The precipitate will be collected, mixed with double distilled water (w/v=1:10) and placed in a water bath maintained at 40°C for 15 minutes with shaking. The mixture was centrifuged at 5000 rpm for 10 minutes and the supernatant will be collected. Ethanol (95%) will be added to the collected supernatant to give a final ethanol concentration of 20% followed by centrifugation at 10000 rpm for 30 minutes, the supernatant will be collected and 95% ethanol will be added until a final ethanol concentration of 50% is reached in order to obtain fucoidan. The centrifugation at 1000 rpm for 30 minutes and dried at 40°C will give pure fucoidan [8].

5.9: Estimation of important functional groups of seaweed polysaccharides

5.9.1: Total Sugar [9].

- Total sugar in the agar samples to be measured by colorimetric method following the method of Dubois using glucose as standard.
- Range of the method: 10-100 µg.
- Sample preparation: 10-20 mg sample to be solubilised in 10 ml of distilled water
- Reagents: Std. Glucose solution: 50 µg AR grade glucose/ml distilled water. AR Grade sulphuric acid
- 5% phenol solution: 5 g AR grade phenol in 100 ml distilled water.
- **Method:** For standard chart, 0.1-1.0 ml aliquots of glucose will be taken. Volume will be made up to 2 ml with distilled water. 1.0 ml 5 % phenol will be added to each of the test tubes. Tubes will be kept in ice bath. 5 ml conc sulphuric acid will be added in one shot to each of the test tubes. Tubes will be cooled. Further the tubes will be incubated at room temperature for 30 min. UV absorbance will be taken at 485 nm.

5.9.2: Test for Galactose and Anhydrogalactose [IS 16147: 2014]

- Boil a mixture of 200 mg of the sample and 20 ml of 10 % sulphuric acid for 3 h. Allow to cool and add excess barium carbonate, mixing with a magnetic stirrer until the solution is pH 7, and filter. Evaporate the filtrate in a rotatory evaporator at 30-50°C under vacuum until a crystalline (or syrupy) residue is obtained. Dissolve in 10 ml of 40 % methanol. This is the hydrolysate. Place 1 to 5 µl spots of hydrolysate on the starting line of two Silica Gel G thin layer plates. On the same plates apply 1 to 10 µg of the reference standards of galactose, rhamnose, galacturonic acid, 3, 6-anhydrogalactose, mannose, arabinose and xylose.
- Develop one plate in solvent A and one plate in solvent B: a) A mixture of formic acid, methyl ethyl ketone, tertiary butanol and water (15/30/40/15 by volume); and b) A mixture of glacial acetic acid/chloroform/water (74/65/11 by volume). After

development spray with a solution of 1.23 g anisidine and 1.66 g phthalic acid in 100 ml ethanol and heat the plates at 100°C for 10 min. A greenish yellow colour is produced with hexoses, a red colour with pentoses and a brown colour with uronic acids. Compare sample spots with those for the solutions of the reference standards and identify the constituents.

5.9.3: Test for 3,6-anhydrogalactose [10] (Alternative method)

- 3,6-anhydrogalactose content of the seaweed samples can be estimated by turbidimetric method described by Yaphe and Arsenault using fructose as standard.
- Range of the method 8µg-32µg. Accurately weighed seaweed samples will be solubilised in distilled water (5 mg/5 ml).
- *Reagents:* 1. Standard Fructose Solution: Stock solution: 27 mg of AR grade fructose in 50 ml of benzoic acid saturated distilled water (Prior to this water was warned to dissolve benzoic acid). Working Solution: 3.0 ml of stock solution to 100 ml with distilled water.
- AcetalSolution : Stock solution : 82 mg acetal (100 µl) in 10 ml distilled water.
- Working solution: 1 ml of stock solution 25 ml with distilled water.
- Resorcinol solution: Stock Solution: 150 mg AR grade resorcinol in 100 ml distilled water
- Resorcinol-acetalreagent: 100 ml concentrated HCl was added to 9.0 ml of resorcinol solution. 1.0 ml working acetal solution will be added.
- **Method:** For standard chart aliquots from 0.5 to 2.0 ml will be taken and volume will be made up to 2.0 ml with distilled water. Tubes will be cooled in icebath. 10 ml of resorcinol -acetal reagent is to be added to each of the tubes which will be kept at 20°C for 4 min. These tubes will be transferred to water bath at 80 °C for 10 min. Further the tubes will be cooled in icebath resulting development of Redish. UV absorbance will be recorded at 555 nm taking a blank containing water of same volume and other reagents. This will give a standard chart. For analyses of the samples, same procedures were applied, this will give concentration of fructose, to convert fructose to 3,6-anhydrogalactose, the values were multiplied by a factor 1.087.

5.9.4: Test for Infrared Absorption- Carrageenan [IS 16147: 2014]

- Disperse 2 g of the sample in 200 ml of 2.5 % potassium chloride solution, and stir for 1 h. Let stand overnight, stir again for 1 h, and transfer into a centrifuge tube. (If the transfer cannot be made because the dispersion is too viscous, dilute with up to 200 ml of the potassium chloride solution.) Centrifuge for 15 min at approximately 1 000 (rpm) g. Remove the clear supernatant, resuspend the residue in 200 ml of 2.5 % potassium chloride solution, and centrifuge again. Coagulate the combined supernatants by adding 2 volumes of 85 % ethanol or isopropanol (Retain the sediment for use as directed below). Recover the coagulum, and wash it with 250 ml of the alcohol. Press the excess liquid from the coagulum, and dry it at 60°C for 2 h. The product obtained is the non-gelling fraction (lambda carrageenan). Disperse the sediment (retained above) in 250 ml of cold water, heat at 90°C for 10 min, and cool to 60 °C. Coagulate the mixture, and then recover, wash, and dry the coagulum as described above. The product obtained is the gelling fraction (kappa- and iota carrageenan). Prepare a 0.2 % aqueous solution of each fraction, cast films 0.5 mm thick (when dry) on a suitable nonsticking surface such as Teflon, and obtain the infrared absorption spectrum of each film. (Alternatively, the spectra may be obtained using films cast on potassium bromide plates, if care is taken to avoid moisture).

5.10: Test for Loss on Drying [IS 16147: 2014]

- Mix the sample well and accurately weigh 1 g of the substance. Reduce the sample to a fine powder. Tare a glass-stoppered, shallow weighing bottle that has been dried for 30 min at 105 °C to constant weight. Transfer the sample into the bottle, replace the cover, and weigh the bottle and the sample. Distribute the sample as evenly as practicable to a depth of about 5 mm. Place the bottle with its contents in the drying chamber, removing the stopper and leaving it also in the chamber and dry the sample at 105 °C to constant weight. Upon opening the chamber, close the bottle promptly and allow it to come to room temperature in a desiccator before weighing.

5.11: Determination of Viscosity [IS 16147 : 2014]

- Disperse an accurately weighed 15 g sample of commercial product into 500 ml of 60 % (w/w) isopropanol/water at room temperature. Stir gently for 4 h. Filter through ash-free filter paper. Discard the filtrate. Wash the material remaining on the filter paper with two 15 ml portions of 60 % isopropanol/water. Dry the material at 105°C to constant weight. Transfer 7.5 g of the dried sample obtained into a tared, 600 ml tall-form (Berzelius) beaker, and disperse with agitation for 10 to 20 min in 450 ml of deionized water. Add sufficient water to bring the final weight to 500 g, and heat in a water bath with continuous agitation, until a temperature of 80°C is reached (20-30 min). Add water to adjust for loss by evaporation, cool to 76-77°C, and heat in a constant temperature bath at 75°C. Pre-heat the bob and guard of a viscometer to approximately 75°C in water. Dry the bob and guard, and attach them to the viscometer, which should be equipped with a No. 1 spindle (19 mm in diameter, approximately 65 mm in length) and capable of rotating at 30 rpm. Adjust the height of the bob in the sample solution, start the viscometer rotating at 30 rpm and, after six complete revolutions of the viscometer, take the viscometer reading on the 0-100 scale. If the viscosity is very low, increased precision may be obtained by using the Brookfield UL (ultra-low) adapter or equivalent. Record the results in centipoises, obtained by multiplying the reading on the scale by the factor given by the Brookfield manufacturer.

5.12: Determination of Sulphate [IS 16147 : 2014]

- Disperse an accurately weighed 15 g sample of commercial product into 500 ml of 60 % (w/w) isopropanol/water at room temperature. Stir gently for 4 h. Filter through ash-free filter paper. Discard the filtrate. Wash the material remaining on the filter paper with two 15 ml portions of 60 % isopropanol/water. Dry the material at 105°C to constant weight.
- Accurately weigh 1 g of the dried matter.
- Transfer the sample to a 100 ml long-neck round-bottom flask. Add 50 ml of 0.2 N hydrochloric acid. Fit a condenser, preferably one with at least 5 condensing bulbs, to the flask and reflux for 1 h. Add 25 ml of a 10 % (by volume) hydrogen peroxide solution and resume refluxing for about 5 h or until the solution becomes completely clear. Transfer the solution to a 600 ml beaker, bring to a boil, and add drop wise 10 ml of a 10 % barium chloride solution. Heat the reaction mixture for 2 h on a boiling water bath. Filter the mixture through ash free slow filtration filter paper. Wash with boiling distilled water until the filtrate is free from chloride. Dry the filter paper and contents in a drying oven. Gently burn and ash the paper at 800°C in a tared porcelain or silica crucible until the ash is white. Cool in a desiccator. Weigh the crucible

containing the ash. Calculate the %age sulphate from the weight in g (*W*₂) of the ash (barium sulphate) using the formula:

$$W_2 \times 100 \times 0.4116 / W_1$$

Where,

*W*₁ = weight of material taken for test, in g; and

*W*₂ = weight of the ash, in g.

5.13: Determination of Total Ash [IS 16147 : 2014]

- Disperse an accurately weighed 15 g sample of commercial product into 500 ml of 60 % (w/w) isopropanol/water at room temperature. Stir gently for 4 h. Filter through ash-free filter paper. Discard the filtrate. Wash the material remaining on the filter paper with two 15-ml portions of 60 % isopropanol/water. Dry the material at 105°C to constant weight.
- Accurately weigh 2 g of the dried sample (*W*₁). Transfer to a previously ignited, tared silica or platinum crucible.
- Heat the sample with a suitable infrared lamp, increasing the intensity gradually, until the sample is completely charred; continue heating for an additional 30 min. Transfer the crucible with the charred sample into a muffle furnace and ignite at about 550°C for 1 h. Cool in a desiccator and weigh. Repeat the ignition in the muffle furnace until a constant weight (*W*₂) is obtained. If a carbon-free ash is not obtained after the first ignition, moisten the charred spot with a 1-in-10 solution of ammonium nitrate and dry under an infrared lamp. Repeat the ignition step.
- Calculate the %age of total ash of the sample = $W_2 \times 100 / W_1$
*W*₁ : mass of the crucible with the material before drying, in g; and
*W*₂: mass of the crucible with the material after drying and after it has come to room temperature in g.

5.14: Determination of Acid Insoluble Ash [IS 16147: 2014]

- Boil the ash obtained with 25 ml of dilute hydrochloric acid for 5 min, collect the insoluble matter on a suitable ash less filter, wash with hot water, ignite at 800 ± 25°C, cool and weigh. Calculate the % of acid insoluble ash from the weight of the sample taken.

5.15: Determination of Acid Insoluble Matter [IS 16147: 2014]

- Disperse an accurately weighed 15 g sample of commercial product into 500 ml of 60 % (w/w) isopropanol/water at room temperature. Stir gently for 4 h. Filter through ash-free filter paper. Discard the filtrate. Wash the material remaining on the filter paper with two 15 ml portions of 60 % isopropanol/water. Dry the material at 105°C to constant weight.
- Transfer 2 g of the dried sample, accurately weighed, into a 250 ml beaker containing 150 ml of water and 1.5 ml of sulphuric acid test solution. Cover the beaker with a watch glass and heat the mixture on a steam bath for 6 h rubbing down the wall of the beaker frequently with a rubber-tipped stirring rod and replacing any water lost by evaporation. Weigh 500 mg of a suitable acid washed filter aid, pre-dried at 105°C for 1 h, to the nearest 0.1 mg, add this to the sample solution and filter through a tared Gooch crucible provided with an asbestos pad. Wash the residue several times with hot water, dry the crucible and its contents at 105°C for 3 h, cool in a desiccator and weigh. The difference between the total weight and the weight of the filter aid plus crucible and pad is the weight of the acid-insoluble matter. Calculate as %.

5.16: Analysis of gelatine [IS 6850:1973]

- Dissolve about 1 g of material in 100 ml of boiling water and allow to cool about 50 °C. To 5 ml of solution add 5 ml of trinitrophenol. No turbidity should appear till 10 minutes.

5.17: Analysis of water insoluble matter [IS 6850:1973]

- To 6.5 g of material add sufficient water to make it 500 g. Boil for 15 minutes and re adjust the original weight. To 100 g of the sample add hot water to make it 200 ml. Heat almost boiling and filter through sintered glass crucible of porosity G4 and G5. Rinse the container with hot water several times. Heat the crucible at 105 °C. Dry and take weight and calculate % of insoluble matter.

5.18: Plant growth hormones (Phyto hormones) [11-12]

- **Method:** Solid samples: Accurately weighed samples will be added to water (1:20 w/v) and will be stirred at room temperature for 12 h. The filtrate will be used for PGH analysis and in case of liquid sample, the liquid will be directly used for the analyses.
- **5.18.1: Extraction of Auxins:** The pH of the extract to be adjusted to 3 by dropwise adding 1N HCl followed by extraction with di ethyl ether (DEE) (x3), the DEE layer to be partitioned with 5% NaHCO₃. The bicarbonate layer was separated and acidified to pH 3.0 by dropwise adding 1N HCl and once again extracted with DEE (x3). Both the DEE layer to be combined and washed with water and dried with anhydrous sodium sulphate followed by evaporation to dryness and then dissolved in minimum amount of methanol (2 mL). Further analysed by HPLC & mass spectrometry (ESI-MS)
- **5.18.2: Extraction of Cytokinins:** The pH of the extract to be adjusted to pH 3 by dropwise adding 1N HCl followed by extraction with dichloromethane (DCM) (x3), which will be collected and saved. The pH of the aqueous layer to be adjusted to 8 with 1N NaOH, followed by extraction with *n*-butanol (x3). The DCM and butanol layers were evaporated to dryness, dissolved the residues in minimum amounts of methanol (2 mL). Further analysed by HPLC & mass spectrometry (ESI-MS).
- **5.18.3: Extraction of Gibberellins:** The pH of the extract to be adjusted to 2.5 by dropwise adding 3.2 N HCl followed by extraction with ethyl acetate (x3), this layer to be saved. The pH of the aqueous layer to be adjusted to 11.0 by dropwise adding 3.75 M NaOH followed by hydrolysis by heating on a water bath at 60°C for 1 h. The pH to be adjusted to 2.5 by adding 1N HCl, followed by partition with equal volumes of ethyl acetate. This ethyl acetate extract to be combined with the previously saved ethyl acetate layer followed by partition with 0.48 M NaHCO₃. The pH of the bicarbonate layer to be adjusted to 2.5 by dropwise adding 1.6 N HCl followed by partition with ethyl acetate. Finally, the ethyl acetate layer to be evaporated to dryness and the residue to be dissolved in minimum volume of methanol (2 mL). Further analysed by HPLC & mass spectrometry (ESI-MS) Standards were prepared in 25 ppm of each in methanol. HPLC conditions applied as mentioned below Shimadzu prominence HPLC system to be used for the analysis of PGH/Rs. The HPLC solvent system to be comprised of A: water/0.1% HCOOH and B: Methanol/0.1%HCOOH. HPLC elution to be carried out using SUPELCO C18H 5µm 250 mm x 4.6 mm column following below method.

Table 5.18: Gradient table for the HPLC analysis

Time (min)	Flow (mL/min)	Volume % (A)	Volume % (B)
0	0.8	70	30
2	0.8	70	30
20	0.8	0	100
22	0.8	0	100
25	0.8	70	30

- Injection volume for all samples and standards to be maintained as 50 μ l. Column heater temperature to be set at 40 °C and UV detection to be performed using UV-DAD detector. HPLC chromatograms were monitored at 205 (for gibberellins) and 254 (for auxins and cytokinins). Data processing to be done using LC Solution TM software provided by the HPLC manufacturer. The presence of respective PGHs to be confirmed by Electrospray mass spectrometry (ESI-MS & ESI-MS/MS) [Q-TOF-LC/MS, model 6545, Agilent Technologies, USA].

5.19: Characteristic FT-IR bands for the important constituents of seaweed

Class of seaweed	Name of constituent	Monomeric unit		Characteristic FT-IR Band (cm^{-1})	
<i>Phaeophyceae</i>	Alginic acid/alginate	D-Manuronic acid	L-Guluronic acid	808	Mannuronic acid
				1080	Guluronic acid
				1400	Alginate (C-O) Symmetric stretching
				1600	Alginate (C-O) asymmetric stretching
	Fucoidan	Fucose			
				1650-1600	carbonyl functional groups from uronic acid
				850 to 820	Sulfate (axial or equatorial)
	Laminarans	1,3 beta D Glucan		1200 - 950	Glucan;
				950 - 750	anomeric region
				1420	(C=O) symmetric stretching
<i>Rhodophyceae</i>	Agar/Agarose	Galactose	3,6-Anhydro galactose	970-975	Galactose
				935	C-O of 3,6 anhydro-D-galactose

	Kappa Carrageenan	Galactose-4-sulphate	3,6-Anhydro galactose	845	C-O-S of axial secondary sulfate on C-4 of galactose
				935	C-O of 3,6 anhydro-D-galactose
				970-975	Galactose
				1240	S=O sulphate ester
	Iota Carrageenan	Galactose-4-sulphate	3,6-Anhydro galactose-2-sulphate	845	C-O-S of axial secondary sulfate on C-4 of galactose
				805	S=O sulphate ester in 3,6-Anhydro galactose-2-sulphate
				935	C-O of 3,6 anhydro-D-galactose
				970-975	Galactose
				1240	S=O sulphate ester
	Lambda carrageenan	Galactose-2-sulphate	Galactose-2,6-di sulphate	845	C-O-S of axial secondary sulfate on C-4 of galactose
				830	Equatorial secondary 2 sulphate
				935	C-O of 3,6 anhydro-D-galactose
				970-975	Galactose
<i>Chlorophyceae</i>	Ulvan	Ulvanobiuronic acid	Ulvanobioses	1385	S=O asymmetric stretching
				1150	S=O symmetric stretching
				1640	C=O symmetric and asymmetric

5.20: Characteristic FT-IR Absorption Band for carrageenan [IS 16147: 2014]

Wave Number (cm ⁻¹)	Molecular Assignment	Absorbance relative to 1050(cm ⁻¹)		
		Kappa	Iota	Lambda
1220-1260	Ester sulphate	0.3-1.4	1.2-1.7	1.4-2.0
928-933	3,6-anhydrogalactose	0.2-0.7	0.2-0.4	0-0.2
840-850	Galactose-4-sulphate	0.2-0.5	0.2-0.4	-
825-830	Galactose-2-sulphate	-	-	0.2-0.4
810-820	Galactose-6-sulphate	-	-	0.1-0.3
800-805	3,6-anhydrogalactose-2-sulphate	0-0.2	0.2-0.4	-

5.21: Spectrophotometric method for quantification of total phenolics in algal extracts [13]

- Extraction:
 - Liquid samples: The algal extracts are to be extracted with 80% methanol. The 1mL of the seaweed biostimulant is extracted with 10 mL of 80% aqueous methanol (extraction ratio :1:10 v/v) overnight at 4°C in dark. The mixture may be thoroughly vortexed. The contents are centrifuged at 12000 × g for 5 minutes at 4°C and the supernatant used for subsequent analysis.
 - Solid samples: Homogenized Algal tissue (0.3 ± 0.005 g) is to be accurately weighed and extracted with 15 ml of 80% aqueous methanol overnight at 4°C in dark. The mixture may be thoroughly vortexed. The contents are centrifuged at 12000 × g for 5 minutes at 4°C and the supernatant used for subsequent analysis.
- Analysis procedure:
 - Aliquot of 0.05 mL of supernatant is to be added to 1 mL of distilled water and 1 mL of 40% Folin- Ciocalteu Reagent in a test tube. The contents of test tube are thoroughly mixed and incubated for 5 minutes. After 5 minutes of incubation, 2N sodium carbonate is to be added followed by heating the test tubes at 50°C for 30 min in a water bath. The absorbance of the solution is then measured using a spectrophotometer at 765 nm after removal from water bath.
 - A standard curve (20-500 mg/L) is prepared with above method using Gallic acid or phloroglucinol and the concentration of phenolic compounds are expressed in gallic acid or phloroglucinol equivalents.

5.21: References

- [1] Tetsujiro Matsushashi and Kaneo Hayashi. Agar Processed from *Gracilaria foliifera* of Florida. *Agr. Biol. Chern.*, 1972, 36, 1543-1552.
- [2] R. D. Villanueva, A. M. M. Sousa, M. P. Gonçalves, M. Nilsson and L. Hilliou. Production and properties of agar from the invasive marine alga, *Gracilaria vermiculophylla* (Gracilariales, Rhodophyta). *J Appl Phycol.* 2010, 22, 211–220
- [3] R. Meena, Kamallesh Prasad, M. Ganesan, A.K. Siddhanta. Preparation of superior quality products from Indian agarophytes. *J Appl. Phycology* 2011, 23, 183-189.
- [4] Craigie, J. S. & Leigh, C. (1978). Carrageenans and agars. In J. A. Hellebust, & J. S. Craigie (Eds.), *Handbook of phycological methods: Physiological and biochemical methods* (pp. 109–131). Cambridge: Cambridge University Press.

- [5] K. Eswaran, P K. Ghosh, A K. Siddhanta, S. Patolia, C. Periyasamy, A S. Mehta, K H. Mody, B K. Ramavat, Kamalesh Prasad, M R. Rajyaguru, S. Kulandaivel, C R K. Reddy, J B. Pandya, A. Tewari. *Integrated method for production of carrageenan and liquid fertilizer from fresh seaweeds*. US 6,893,479
- [6] Swee-Yong Chee, Ping-Keong Wong, Ching-Lee Wong. Extraction and characterisation of alginate from brown seaweeds (Fucales, Phaeophyceae) collected from Port Dickson, Peninsular Malaysia. *J ApplPhycol*. 2011, 23,191–196
- [7] Kamalesh Prasad, PratyushMaiti, ChandrakantMukesh, Krishna KantaGhara, RamavatarMeena, Subhash Chandra Ghosh. A zero liquid discharge process for the production of alginic acid and its derivatives from *alginophytes*. Indian Patent Application No. 201711025753 dated 20-07-2017
- [8] Thomas Hahn, Siegmund Lang, Roland Ulber, Kai Muffler. Novel procedures for the extraction of fucoidan from brown algae. *Process Biochemistry*, 2012, 47, 1691-1698
- [9] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith. Colorimetric Method for Determination of Sugars and Related Substances. *Anal Chem*. 1956, 28,350-356.
- [10] W. Yaphe, G.P. Arsenault. Improved resorcinol reagent for the determination of fructose, and of 3,6-anhydrogalactose in polysaccharides. *Anal. Biochem*. 1965,13, 143-148.
- [11] Kamalesh Prasad, Arun K. Das, Mihir D. Oza, HarshadBrahmbhatt, Arup K. Siddhanta, RamavatarMeena, K. Eswaran, M.R Rajyaguru and Pushpito K. Ghosh. Detection and quantification of some plant growth regulators in a seaweed-based foliar spray employing a mass spectrometric technique sans chromatographic separation. *J Agr. Food Chem*. 2010, 58 (8), 4594–4601.
- [12] Arun Kumar Das and Kamalesh Prasad. Extraction of plant growth regulators present in *Kappaphycusalvarezii* sap by imidazolium based ionic liquids: Detection and quantification by HPLC–DAD technique. *Analytical Methods*, 2015, 7, 9064–9067
- [13] C.W.F. Fu, C.W. Ho, W.T.L. Yong, F. Abas, T.B. Tan, C.P. Tan. Extraction of phenolic antioxidants from four selected seaweeds obtained from Sabah. *International Food Research Journal* 2016, 23(6), 2363-2369

Chapter 6: Suggestive List of equipment for Biostimulant Quality Control and Testing Laboratory

6.1: High Performance Liquid Chromatography (HPLC):

- As per FCO Provisions it is necessary to analyze Neem Oil Content in Urea. For analysis of Neem Oil Components i.e. Azadirachtin, Nimbin, Salannin, Meliacinetc; HPLC is only prescribed/notified method under Fertilizer Control Order, 1985. HPLC is also required for analysis of other components like Amino Acids, Vitamins, Antioxidants etc. in Bio-Stimulants.

6.2: Liquid Chromatography - Tandem Mass Spectrometry (LC-MS-MS):

- Required for qualitative and quantitative estimation of contaminants in Bio-stimulants i.e. Pesticides, Mycotoxins, Anti-biotic etc, residue analysis with user friendly software to meet the National/Global regulations.

6.3: Gas Chromatography-Tandem Mass Spectrometry (GC-MS/MS):

- Required for qualitative and quantitative estimation of contaminants in Bio-stimulants i.e. Pesticides, Mycotoxins, Anti-biotic etc, residue analysis with user friendly software to meet the National/Global regulations.

6.4: Inductively Coupled Plasma Spectrophotometer (ICP):

- To analyze components in different fertilizers notified under FCO, 1985 i.e., Silicon in Silicon Di-oxide etc. includes other parameter in Bio-stimulants. We can measure the value upto ppb and applicable to analyze the percent of metals if present as traces also.

6.5: Thermo Gravimetric Analysis System (TGA):

- To be used to identify thermal effects towards change of mass of a sample with temperature or time during temperature rise, constant temperature or temperature reduction. It is also to be used to characterize materials with regard to their composition.

6.6: Fourier Transform Infrared Spectrophotometer (FTIR):

- FTIR analysis used to characterize/identification both organic and inorganic evidence in bio-stimulants/products. In this analysis low amount can be measured within minimum time. It is also helpful to identify compounds, impurities and functional groups in qualitative analysis includes identification & structural analysis of chemical compounds.

6.7: High-performance thin layer chromatography:

- High-performance thin layer chromatography is one of the sophisticated instrumental techniques based on the full capabilities of thin layer chromatography. The advantages of automation, scanning, full optimization, selective detection principle, minimum sample preparation, hyphenation, and so on enable it to be a powerful analytical tool for chromatographic information of food stuffs. It is also ideally suitable for the analysis of botanical/plant extracts including other components i.e., vitamins etc.

6.8: Others:

- Reciprocal Shaker
- Rocker Shaker
- High Speed Refrigerated Centrifuge