# Generally Recognized As Safe Notification

## *Chitosanase from Bacillus subtilis* CSSC

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# List of Abbreviations

| | |
|---|---|
| ADI | Advisable Dietary Intake |
| bw | body weight |
| CAS | Chemical Abstracts Service |
| cGMP | current Good Manufacturing Practice |
| EC | Enzyme Commission |
| EDI | Estimated Dietary Intake |
| EFSA | European Food Safety Authority |
| FDA | US Food and Drug Administration |
| g | gram |
| GMO | Genetically Modified Organism |
| IUBMB | International Union of Biochemistry and Molecular Biology |
| JECFA | Joint WHO/FAO Expert Committee on Food Additives |
| WHO/FAO | World Health Organization/Food and Agriculture Organization (UN) |
| kDa | kilodalton |
| kg | kilogram |
| mg | milligram |
| ml | milliliter |
| l | liter |
| MoS | Margin of Safety |
| NOAEL | No Observed Adverse Effect Level |
| ppm | parts per million |
| TOS | Total Organic Solids |
| U | Unit |
| WSC | Water-soluble Chitosan |

# Part 1. 21 CFR 170.225: Signed Statements and Certification

## 1.1 GRAS Notice Submission

Advanced Enzyme Technologies Ltd. (hereinafter "Advanced Enzymes" submits this GRAS notice in accordance with 21 CFR part 170, subpart E.

## 1.2 Name and Address of Notifier

| | |
|---|---|
| Name: | Advanced Enzyme Technologies Ltd. |
| Address: | 5th Floor, 'A' wing, Sun Magnetica LIC Service Road, Louiswadi |
| Postal code and City: | Thane (W) 400604 |
| State: | Maharashtra |
| Country: | India |
| Tel. no: | +91 22 41703200 |
| Fax no: | +91 22 25835159 |
| E-mail: | info@advancedenzymes.com |

**Person Responsible for the dossier**

| | |
|---|---|
| Name: | Dr. Anil Kumar Gupta,<br>VP – Research & Development |
| Address: | Advanced Enzyme Technologies Ltd. 5th Floor, 'A' wing, Sun Magnetica LIC Service Road, Louiswadi |
| Postal code and City: | Thane (W) 400604 |
| State: | Maharashtra |
| Country: | India |
| Tel. no: | +91 22 25830284 |
| E-mail: | anil@advancedenzymes.com |

**Agent who is authorized to act on behalf of the Notifier:**

Kevin O. Gillies
Kevin O. Gillies Consulting Services, LLC
info@kogilliesconsultingservices.com

## 1.3 Name of Notified Substance

The common and usual name of the notified substance is 'Chitosanase', produced by a non-genetically modified *Bacillus subtilis* CSSC.

Chitosanase (IUBMB E.C. 3.2.1.132, CAS No. 51570-20-8,

Systematic name: chitosan N-acetylglucosaminohydrolase

In this GRAS Notice, the Chitosanase from *Bacillus subtilis* CSSC is referred to by names such as 'Chitosanase', 'Chitosanase from *Bacillus subtilis* (strain CSSC)'. 'Chitosanase from *Bacillus subtilis subspecies subtilis.'* *Bacillus subtilis* CSSC is a propriety strain of M/s Advanced Enzyme Technologies Ltd. "CSSC" is the strain designation.

## 1.4 Intended Conditions of Use

Chitosanase from *Bacillus subtilis* CSSC is intended to be used as a processing aid[1] in chitosan hydrolysis. The resultant hydrolysate Water Soluble Chitosan (WSC) is intended to be used as a preservative in the food industry, more specifically in the preservation of shrimp.

Chitosanase produces enzymatically hydrolyzed WSC, which *inter alia*, preserves properties (e.g. moisture content, color, texture etc.) of shrimp during storage and improves product shelf life. WSC can substitute for conventional chemical preservatives in this application. (Yu Guang-li et al. 1996; Chouljenko et al. 2016).

The enzyme Chitosanase (E.C. 3.2.1.132) in this application is used as a processing aid at a usage rate of 2.55 g TOS/kg raw material to produce WSC. Once the substrate chitosan is depleted, the enzyme does not have a technical role in the final food.

Advanced Enzymes' studies suggest that approximately 3 g (i.e. 2.55 g TOS/kg) of the Chitosanase (approx. 2000U/g) enzyme is required to convert one kg chitosan to WSC (i.e. 3000 ppm)[2]. Available data suggest a dose of 5 g WSC for preservation of 1 kg of shrimp. Assuming that all the enzyme used in reaction remains in the WSC, then the maximum quantity of the Chitosanase in shrimp will be 15 ppm[3] (i.e. 12.74 mg TOS/kg)[4]. Considering an average of 19.26 g seafood consumption per person by US population and the assumption that all seafood consumed in the US is in the form of WSC-treated shrimp, the maximum daily intake of Chitosanase will be 0.245 mg TOS/person/day.

## 1.5 Statutory Basis for GRAS Status

Advanced Enzymes has determined that the intended use of Chitosanase from *Bacillus subtilis* CSSC is GRAS through scientific procedures in accordance with 21 CFR §170.30(a) and (b).

---

[1] 21CFR100.100 (a)(2)(ii)(c); "(c) Substances that are added to a food for their technical or functional effect in the processing but are present in the finished food at insignificant levels and do not have any technical or functional effect in that food".

[2] Advanced Enzymes; considering, maximum 3 g chitosanase used for hydrolysis 1kg of Chitosan i.e. 3000mg/kg OR 3000 ppm

[3] 5g/L of water-soluble chitosan is applied to 1 kg shrimp for the preparation of frozen shrimp to extend the shelf life and quality. Therefore (5/1000 x 3) = 0.015 g/kg i.e. 15mg/kg OR 15 ppm chitosanase is present in the 1 kg frozen shrimp

[4] TOS of Chitosanase is estimated as 84.92%

Total organic solids (TOS) is the sum of all organic compounds present in the enzyme preparation derived from the enzyme source and manufacturing process. TOS is calculated as follows:

TOS (%) = 100 – (A + W + D), where: A = % ash, W = % water, D = % diluents and other formulation ingredients.

## 1.6 Premarket Exempt Status

Since Advanced Enzymes has determined that the intended use of Chitosanase from *Bacillus subtilis* CSSC is GRAS, the use of the notified substance is exempt from pre-market approval requirements of the Federal Food, Drug, and Cosmetic Act.

## 1.7 Data Availability

Advanced Enzymes agrees to make the data and information that are the basis for the determination of GRAS status available to FDA upon request. Such data and information may be sent by Advanced Enzymes. to FDA either in electronic format or on paper or reviewed during customary business hours at Advanced Enzyme Technologies, Ltd., 4880 Murrieta Street, Chino, CA 91710.

## 1.8 FOIA Statement

None of the data and information in this GRAS notice is exempt from disclosure under the Freedom of Information Act, 5 U.S.C. §552.

## 1.9 Certification

To the best of Advanced Enzymes' knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to the company and pertinent to the evaluation of the safety and GRAS status of the intended use of Chitosanase from *Bacillus subtilis* CSSC.

## 1.10 FSIS Statement

Not applicable.

## 1.11 Signature of Responsible Party or Agent

Kevin O. Gillies

Kevin O. Gillies Consulting Services, LLC
info@kogilliesconsultingservices.com
December 8, 2020

# Part 2. 21 CFR 170.230: Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

## 2.1 Identity/ Identification

### *2.1.1 SCIENTIFIC NAME, TAXONOMY AND OTHER NAMES*

| | |
|---|---|
| Name of the enzyme: | Chitosanase |
| Systematic name: | Chitosan *N*-acetylglucosaminohydrolase |
| EC (IUBMB) number: | 3.2.1.132 |
| CAS No. | 51570-20-8 |
| Reaction catalyzed: | Hydrolysis of β-1,4 linkages between D-glucosamine residues in a partly acetylated chitosan. |

The classification of the enzyme according to the IUBMB is as follows:

| | |
|---|---|
| EC 3 | Hydrolases |
| EC 3.2 | Glycosylases |
| EC 3.2.1 | Glycosidases i.e. enzymes hydrolyzing O- and S-glycosyl compound |
| EC 3.2.1.132 | Chitosanase |

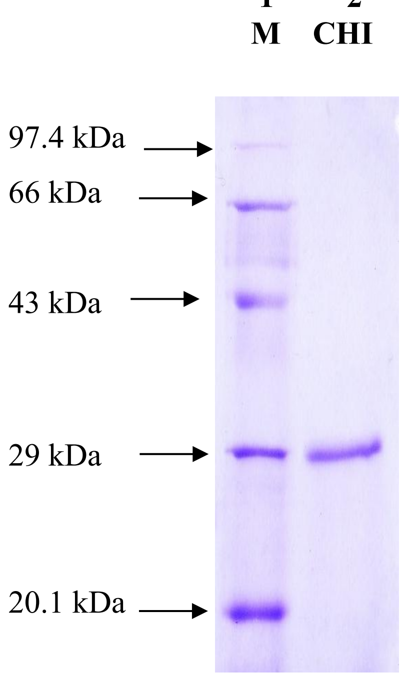
### *2.1.2 AMINO ACID SEQUENCE AND MOLECULAR WEIGHT OF ENZYME PROTEIN*

Chitosanase from *B. subtilis* CSSC is comprised of 277 amino acids having following sequence

MKISMQKADFWKKAAISLLVFTMFFTLMMSETVFAAGLNKDQKRRAEQLTSIFENGTT
EIQYGYVERLDDGRGYTCGRAGFTTATGDALEVVEVYTKAVPNNKLKKYLPELRRLA
KEESDDTSNLKGFASAWKSLANDKEFRAAQDKVNDHLYYQPAMKRSDNAGLKTALA
RAVMYDTVIQHGDGDDPDSFYALIKRTNKKAGGSPKDGIDEKKWLNKFLDVRYDDL
MNPANHDTRDEWRESVARVDVLRSIAKENNYNLNGPIHVRSNEYGNFVIK

Molecular weight of the Chitosanase from *B. subtilis* CSSC was determined as 29 kDA (kilodalton) following SDS-PAGE (see Figure 1), which agrees with the molecular weight obtained with a gel permeation chromatography method, indicating that the enzyme is a monomer. Gel permeation chromatography was performed on a HiLoad 16/600 Superdex 200 pg gel filtration column.

**Figure 1. SDS PAGE analysis of Chitosanase derived from B. subtilis CSSC**




Lane 1- M: Molecular Weight Marker Lane 2- CHI: Chitosanase
Fig -1 Electrophoresis of Chitosanase preparation from *B. subtilis* CSSC

### *2.1.3 ENZYME ACTIVITY*

Chitosanase (IUBMB 3.2.1.132) catalyzes the hydrolysis of β-1,4 linkages between D-glucosamine residues in a partly acetylated chitosan (Thadathil et al. 2014). Enzyme activity was measured at pH 6.0 and 40°C using chitosan as the substrate. One unit of enzyme activity is defined as the amount of enzyme that produces one micromole of reducing sugars, measured as glucosamine equivalents per minute under the given assay conditions.

To the best of our knowledge, the Chitosanase from *B. subtilis* CSSC described in this dossier does not possess any enzymatic side reaction activities which might cause adverse effects.

### 2.1.3.1 Information on Chitosanase pH and temperature optima

The activity of Chitosanase from *B. subtilis* CSSC was measured under various pH and temperature conditions.

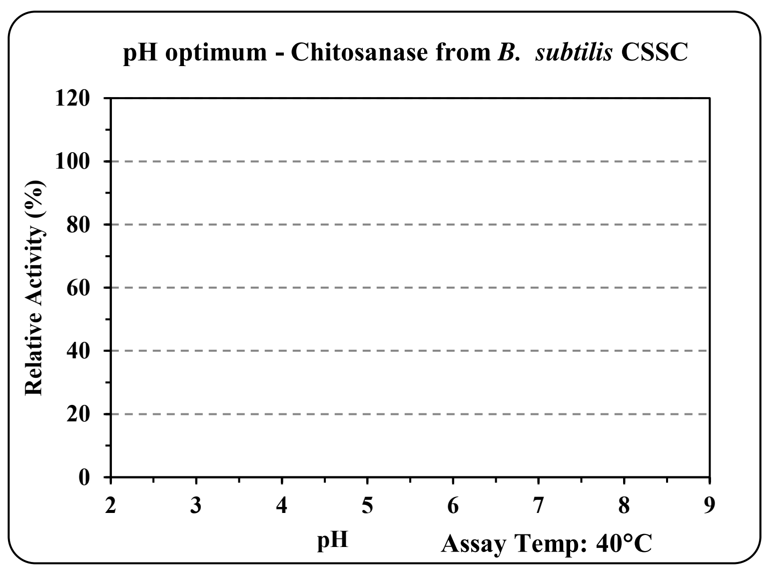



**Fig: 2: pH Optimum of Chitosanase from *B. subtilis* CSSC**

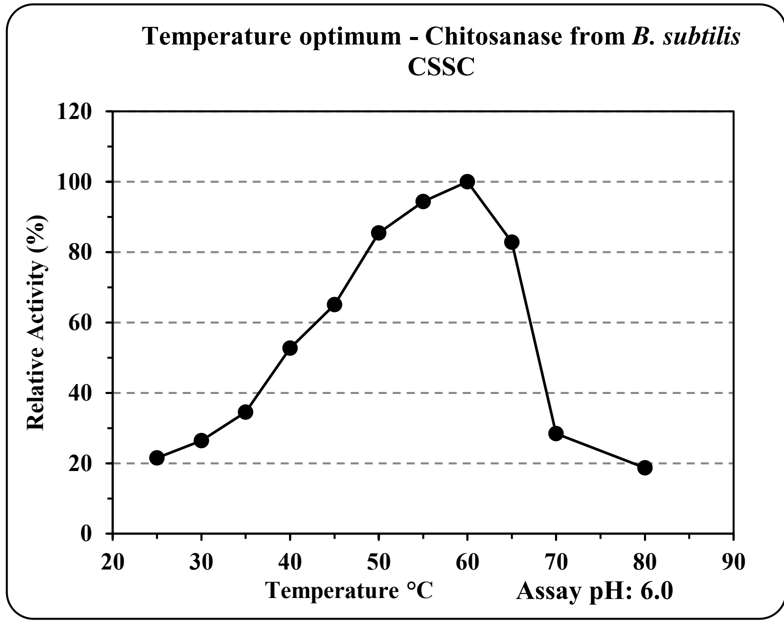



**Fig: 3: Temperature Optimum of Chitosanase from *B. subtilis* CSSC**

The Chitosanase exhibited activity between pH 4.0 to 8.0 and temperature 25°C to 80°C, with pH 7.0 and temperature 80°C as optimum for activity.

### 2.1.3.2 Information on Chitosanase stability

The stability of the food enzyme Chitosanase from *B. subtilis* CSSC was measured under various pH and temperature conditions.

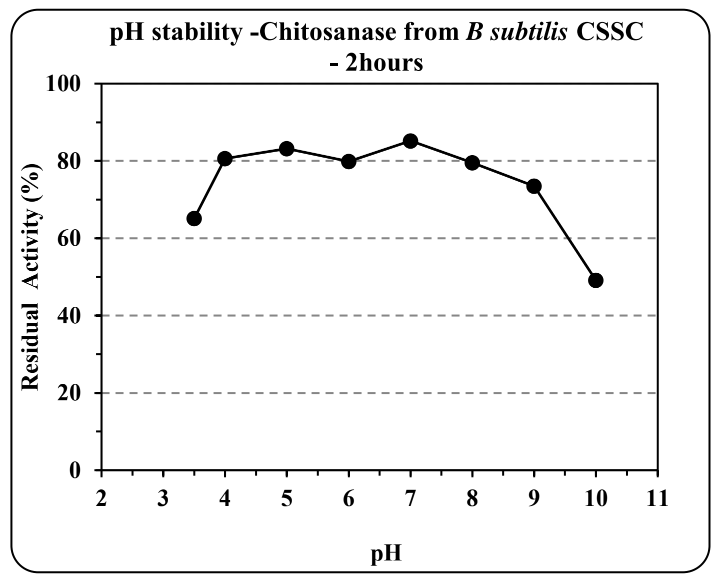



**Fig: 4: pH stability of Chitosanase from *B. subtilis* CSSC**

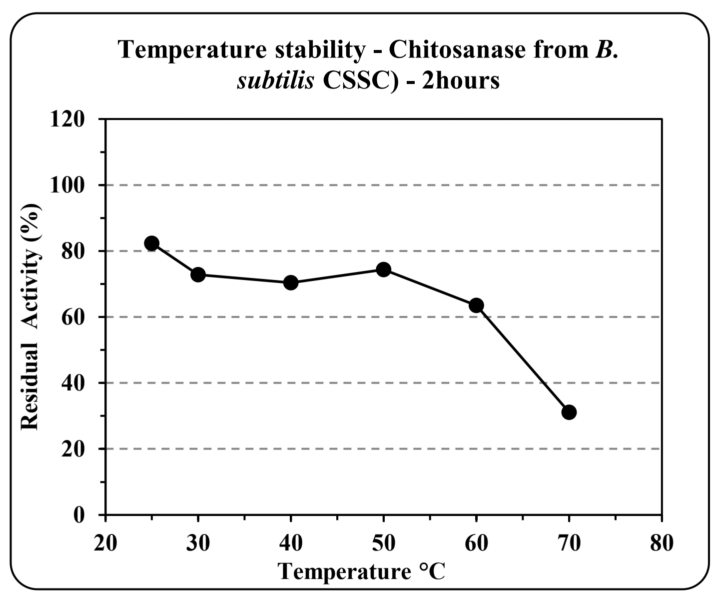



**Fig: 5: Temperature stability of Chitosanase from *B. subtilis* CSSC**

The Chitosanase from *B. subtilis* CSSC retained 80% activity when incubated between pH 4.0 to 8.0 for 2 hrs. The activity declined rapidly after pH 9.0 (Figure 4). The enzyme was found stable up to 50°C, the stability declined sharply after 60°C (Figure 5).

## 2.2 Production organism

### *2.2.1 SCIENTIFIC NAME, TAXONOMY AND OTHER NAMES*

The microorganism used for the production of the Chitosanase preparation that is the subject of this Notice, is a non-genetically modified organism (GMO) *Bacillus subtilis* subsp. *subtilis* CSSC.[5]

Identification of the CSSC strain was carried out using both 16S rDNA and the gyrB gene as phylogenetic markers, as closely related *Bacillus* species cannot be distinguished by 16S rRNA sequence analysis alone. The CSSC strain was identified as *Bacillus subtilis* subsp. *subtilis* by BLAST analysis of the 16S rDNA and gyrB sequences.

**Taxonomy:**

| | | |
|---|---|---|
| Phylum | : | Firmicutes |
| Class | : | Bacilli |
| Order | : | Bacillales |
| Family | : | Bacillaceae |
| Genus | : | *Bacillus* |
| Species | : | *subtilis* subspecies *subtilis* |

Prior to 1952, '*Bacillus subtilis*' was a name given to all aerobic endospore-forming bacilli (Logan, 1988). Numerous species that appeared in the early literature are no longer recognized as official species. Former species designations that are now considered to be members of the species *Bacillus subtilis* include *B. aterrimus*, *B. mesentericus*, *B. niger*, *B. panis*, *B. vulgarus*, *B. nigrificans*, and *B. natto* (cited in Logan, 1988).

### *2.2.2 PRODUCTION STRAIN*

The microorganism used for the production of Chitosanase is a non-pathogenic, nontoxigenic, non-genetically modified strain *B. subtilis* CSSC. The production strain was assessed for its toxigenic potential as recommended by EFSA, 2014. *In vitro* cytotoxicity test of *B. subtilis* CSSC was carried out on Vero cells using PI based fluorimetric assay. The strain *B. subtilis* CSSC was found to be non-cytotoxic. (Refer to section 6.1 for details).

*B. subtilis* CSSC was tested for enterotoxin production by Duopath® Cereus Enterotoxins test kit (Merck) and found negative for enterotoxins. *B. subtilis* CSSC was examined for the absence of hemolysin, hbl; nonhemolytic, nhe; cytotoxin, cytK and emetic toxin cereulide, ces producing genes using the polymerase chain reaction (PCR) method. The absence of PCR products for the toxin genes confirms the absence of these toxins in *B. subtilis* CSSC.

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[5] *Bacillus subtilis* subsp. *subtilis*. CSSC, *Bacillus subtilis* CSSC and *B. subtilis* CSSC are used interchangeably while referring to the production strain, in certain sections of the Notice.

The production strain *B. subtilis* CSSC was also assessed for its toxigenic potential by cytotoxicity test using Vero cells (EFSA, 2014). The test is based on the principle that the DNA intercalating agent propidium iodide will stain DNA of cells having leaky cell membranes as a result of treatment with a cytotoxic agent, thereby enhancing the resulting intracellular fluorescent signal. The DNA of intact cells would not show any uptake of propidium iodide, resulting in basal level, negligible fluorescence, whereas the DNA of the dead cells can be stained and measured. The fluorescence of the live cells was measured by lysing the cells with 0.2% triton X 100.

20 µg Doxorubicin (DOX) was used as positive control for the cytotoxicity assays which gave the T/C% of 4.81 unlike the negative control (only cells in the media without any active agent) which gave T/C% of 100.

The study indicated that the sample of *B. subtilis* CSSC did not elicit cytotoxicity on Vero cells (Table 4).

**Table 1: Test for Detection of Cytotoxicity Using Vero cells**

| Test Article | Fluorescence Units in Live Cells | (T/C%)* |
|---|---|---|
| Background | 0 | 0 |
| Positive control | 820 | 4.82 |
| Negative control | 17014 | 100.00 |
| *B. subtilis* CSSC – 10 µl | 12981 | 76.30 |
| *B. subtilis* CSSC – 50 µl | 12488 | 73.40 |
| *B. subtilis* CSSC – 100 µl | 11262 | 66.19 |

* The measured effect of the test article is expressed in terms of survival of Vera cells, obtained by comparison of the mean number of fluorescence units (FU) of live cells in the treated groups with the untreated controls (test verses control value [T/C]). A test article is active if it reduced the FU of viable cells in the test sample to less than 30% of the control group value (T/C% ≤ 30%). Cytotoxicity of *B. subtilis* CSSC at three different concentrations was measured in triplicate.

## 2.3 Manufacturing process

### *2.3.1 OVERVIEW*

Chitosanase is produced by a batch fermentation process employing a non-GMO *B. subtilis* CSSC. Fermentation is carried out in accordance with current Good Manufacturing Practice (cGMP) and the principles of Hazard Analysis and Critical Control Points (HACCP).[6] The manufacturing facility is ISO 9001, ISO 22000 and cGMP certified.

Fermentation is a well-known process that occurs in food and has been used for the production of food enzymes for decades. Liquid state or submerged fermentation is used to produce the Chitosanase. The typical fermentation batch size ranges from 100 L to 50,000 L.

As shown below, the key steps for production of Chitosanase are fermentation, recovery, formulation, and packaging. The process is illustrated in Figure 1.

[6] 21 CFR 110 and 117

### *2.3.2 FERMENTATION*

#### 2.3.2.1 Raw materials

All raw materials used in the fermentation process are food grade and are either approved food additives or GRAS ingredients.

#### 2.3.2.2 Inoculum (Seed)

A suspension of a pure culture of *B. subtilis* CSSC is aseptically transferred to an inoculum flask containing fermentation medium.

The culture is grown in the flask under optimum conditions to obtain a sufficient amount of biomass, which is subsequently used as inoculum for the seed fermentation.

#### 2.3.2.3 Seed Fermentation

The inoculum is aseptically transferred to the seed fermenter containing seed fermentation medium. When a sufficient amount of biomass is developed (typically up to 16 hours), the content of the seed fermenter is used for inoculation of the main fermentation.

#### 2.3.2.4 Main fermentation

The enzyme production takes place at this stage. The fermentation is operated in batch mode. The production microorganism *B. subtilis* CSSC grown in seed fermenter is aseptically transferred to the main fermenter containing the sterilized and cooled fermentation medium. The fermentation process is continued for a predetermined time or until laboratory test data show that the desired enzyme production has been obtained or that the rate of enzyme production has decreased below a predetermined production rate. When the desired enzyme level is obtained, the fermentation is complete.

The samples are checked microscopically during seed and fermentation stages at regular intervals for contamination with other microorganisms. Microscopic observations for purity are also confirmed by plating the samples on nutrient agar and incubating plates at 37˚C for 24 hrs. A production batch is stopped and discarded once any contamination is observed at any stage.

### *2.3.3 RECOVERY*

The Chitosanase produced in the fermentation process is separated from the biomass and other insoluble at this stage.

The steps of enzyme recovery include:

- Primary separation (biomass and insoluble/unutilized media from liquid)
- Concentration
- Pre-filtration and micro (germ) filtration
- Spray drying

#### 2.3.3.1 Primary Separation

Filter aids are added to biomass flocs and filtration is carried out at a controlled pH using a horizontal filter press. This enables separation of the biomass and other insoluble from the

enzyme containing liquid. The primary separation is performed at defined pH and temperature ranges in order to minimize the loss of enzyme activity.

#### 2.3.3.2 Concentration

The primary filtration step is followed by ultrafiltration and diafiltration to concentrate the enzyme containing liquid and to reach the desired enzyme activity. Temperature and pH are controlled during this step.

#### 2.3.3.3 Pre-filtration and micro-filtration (Germ filtration)

A filtration step on a dedicated micro (germ) filtration media is performed to ensure the removal of the production strain cells and fine insoluble. The concentrated enzyme liquid obtained after microfiltration, if required, is stabilized using suitable stabilizer such as glycerol.

#### 2.3.3.4 Spray Drying

The concentrated enzyme solution is spray-dried in presence of approved food-grade stabilizers (e.g., maltodextrin) to obtain the unformulated concentrate.

## *2.3.4* Formulation and Packaging

Chitosanase is sold as a powder preparation.

If required, the spray-dried unformulated concentrate powder (not less than 2,000 U/g) is further formulated with approved food grade formulating agents such as maltodextrin and adjusted to a declared enzyme activity.

The Chitosanase preparation is tested by Quality Control for the quality specifications and released by Quality Assurance. The final product is packed in suitable food packaging material before storage. Warehousing and transportation are performed according to specified conditions mentioned on the accordant product label for final preparations.

Preparation of laboratory seed
↓
Preparation of seed vessel ← Check for purity of microorganism pH, temperature
↓
Preparation of production medium
↓
Fermentation running cycle ← Check for purity of the production microorganism, pH, activity during fermentation process
↓
Harvesting of batch
↓
Filtration ← Check for pH and enzyme activity
↓
Concentration ← Check for pH and enzyme activity
↓
Filtration ← Check for pH and enzyme activity
↓
Microfiltration ← Check for pH and enzyme activity
↓
Spray drying ← Appearance, enzyme activity and other tests as per product specification
↓ ← QA release
Packing & labeling & Dispatch

**Fig.6: Manufacturing process for Chitosanase of *B. subtilis* CSSC**

## 2.4 Product Specifications and Compositional Variability

### *2.4.1 PRODUCT SPECIFICATIONS*

Chitosanase complies with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (FAO/WHO, 2006), which are part of the product release specifications for Chitosanase preparation established by Advanced Enzyme Technologies Ltd. and summarized in Table 2. All methods have been validated for this purpose.

**Table 2: Product Specifications for Chitosanase from *B subtilis* CSSC**

| Product specification | Advanced Enzyme Technologies Ltd. | |
|---|---|---|
| | Limits | Reference Method |
| Chitosanase activity | Not less than 2,000 U/g | Internal method |
| Appearance/ Description | Light brown to brown colored powder with characteristics odor | Visual, olfactory |
| Moisture/ Loss on Drying | Not more than 10.0% | AOAC 926.08 |
| Solubility | Soluble in water | Internal method |
| Heavy metals | Not more than 30.0 ppm | AOAC 984.27 |
| Lead | Not more than 5.0 ppm | AOAC 984.27 |
| Total viable count | Not more than 10000 cfu/g | Harmonized method (IP, BP, EP and USP) |
| Total coliform | Not more than 30 cfu/g | FDA Bacteriological Analytical Manual |
| *E. coli /25g* | Negative by test | Harmonized Pharmacopoeial method (EP, BP, USP, and IP) |
| *Salmonella* spp. /25g | Negative by test | Harmonized Pharmacopoeial method (BP, USP and IP) |
| *Antimicrobial activity* | Absent by test | JECFA 2003 (FNP 52, Add. 11) |

### *2.4.2 COMPOSITIONAL VARIABILITY*

Commercial enzymes, whether used in the production of food, feed or for technological purposes, are biological isolates of variable composition. Apart from the enzyme protein in question, microbial food enzymes also contain some substances derived from the producing micro-organism and the fermentation medium. These constituents consist of organic materials and inorganic salts. As established by JECFA (FAO/WHO, 2006), the percentages of these organic materials are summarized and expressed as Total Organic Solids (TOS) .[7]

#### 2.4.2.1 Quantitative Composition

Relative purity of Chitosanase from *B. subtilis* CSSC was measured, and the TOS values were calculated, in 3 batches after drying.

**Table 3: Analysis of Compositional Variability of Chitosanase from *B. subtilis* CSSC**

| Batch no | 061434[8] | 101423 | 101435 | Mean enzyme activity |
|---|---|---|---|---|
| Ash (%) | 7.76 | 8.29 | 7.30 | - |
| Water (%) | 7.25 | 7.48 | 7.10 | - |
| TOS (%) | 84.99 | 84.23 | 85.42 | - |
| Chitosanase activity (U/g) | 2875 | 2648 | 2990 | 2837.66 |
| U/mg TOS | 3.38 | 3.14 | 3.50 | - |
| Unit Definition: One unit of activity is defined as the amount of enzyme that produces one micromole of reducing sugars, measured as glucosamine equivalents per minute under the given conditions of the assay. | | | | |

The enzyme activity was measured by in-house validated methods. Ash and dry matter content were determined using standard methods.

[7] The TOS value is an internationally accepted method to describe the chemical composition of commercial food enzymes. % TOS = 100 –(A+W+D) where A= % ash, W= % Water and D = % Diluents/other ingredients. The ratio between the enzyme activity (U) and TOS (g) is an indication of the relative purity of the enzyme.
[8] The batch used for toxicity studies adheres to JECFA specifications for food enzyme(s).

### 2.4.2.2 Data on batch to batch variability for relevant parameters

Three batches of Chitosanase from *B. subtilis* CSSC were analyzed and the results compared with the specifications. As shown in Table 3, all tested batches complied with the product specifications, demonstrating suitability of the production process.

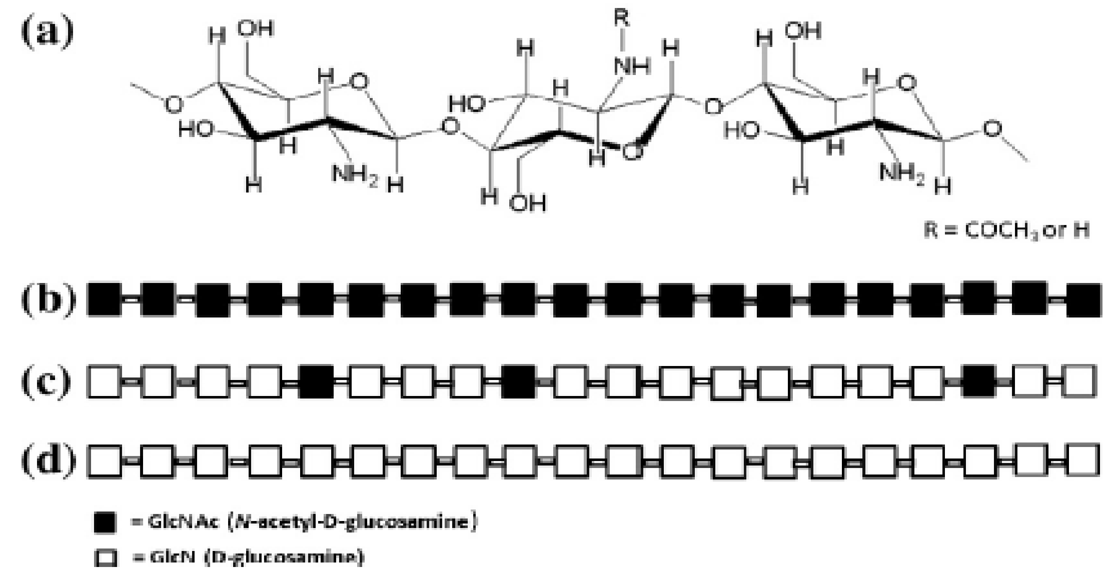
**Table 4: Analysis of Compositional Variability of Chitosanase from *B. subtilis* CSSC**

| Parameter | Specification | Batch | | |
|---|---|---|---|---|
| | | 061434 | 101423 | 101435 |
| Chitosanase Activity | Not less than 2000 U/g | 2875 U/g | 2648 U/g | 2990 U/g |
| Description | Light brown to brown colored powder with characteristic odor | Light brown colored powder with characteristic odor | Light brown colored powder with characteristic odor | Light brown colored powder with characteristic odor |
| Solubility | Soluble in water | Soluble in water | Soluble in water | Soluble in water |
| Moisture/Loss on drying (%) | Not more than 10.0% | 7.25% | 7.48 % | 7.10 % |
| Heavy Metal Analysis | | | | |
| Heavy metals | Not more than 30 ppm | Complies | Complies | Complies |
| Lead | Not more than 5.0 ppm | Complies | Complies | Complies |
| Microbial Analysis | | | | |
| Total viable count | Not more than 10000 cfu/g | Complies | Complies | Complies |
| Total Coliform | Not more than 30 cfu/g | Complies | Complies | Complies |
| *E. coli* | Absent in 25g | Complies | Complies | Complies |
| *Salmonella* spp. | Absent in 25g | Complies | Complies | Complies |
| *Antimicrobial activity* | Absent by test | Complies | Complies | Complies |

## 2.5 Chitosanase Technical Effect

### *2.5.1 MODE OF ACTION*

Chitosanase catalyzes hydrolysis of β-(1→4)-linkages between D-glucosamine residues in a partly acetylated chitosan. (Thadathil et al. 2014)




(a) Chemical structure of chitosan
(b) Distribution of N-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) in chitin
(c) Partially N-deacetylated chitosan
(d) Fully N-deacetylated chitosan

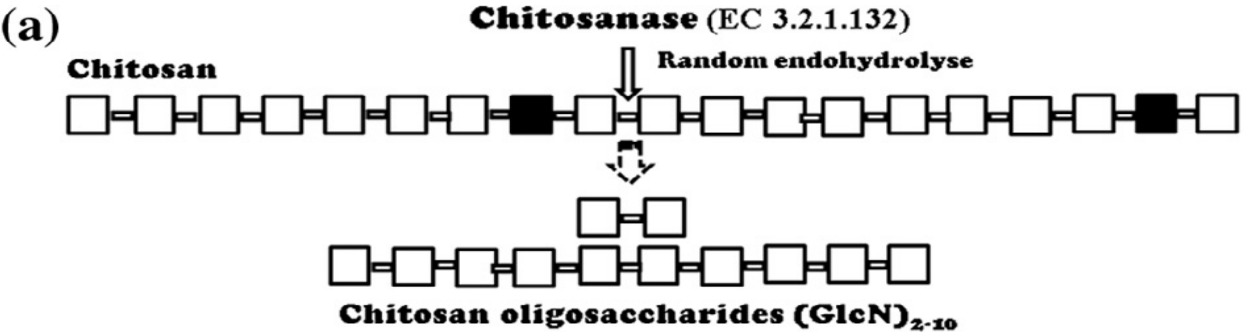



**Fig. 7: Chitosanase, mode of action**

### *2.5.2 USES*

Chitosanase enzyme hydrolyzes β-1,4 linked D-glucosamine residues in the chitosan molecules (Izume et al. 1992) resulting in the production of enzymatically hydrolyzed WSC which is used as a preservative in the food industry. WSC is reported to be used in preservation and storage of

food, more specifically in the preservation of shrimp. (Yu Guang-li et al. 1996; Chouljenko et al. 2016).

Shrimp is one of the most consumed sea foods worldwide. Storage and preservation of shrimp poses a challenge to the food industry, as shrimp spoils when stored at refrigeration temperatures it is a necessity to freeze the shrimp for storage. The shelf life of the frozen shrimp can be increased by using water-soluble chitosan as preservative. (Chouljenko et al. 2016).

WSC has the ability to penetrate into the shrimp muscle tissue. It is known to lower the aerobic plate counts, yeast and mold counts, and also prevent lipid oxidation. It is observed that the shrimp treated with water-soluble chitosan retain their initial moisture content, color, and texture during storage. (Yu Guang-li et al. 1996; Chouljenko et al. 2016)

The process flow chart of the production of enzymatically hydrolyzed WSC and its subsequent application in preservation of frozen shrimp is shown below. The chart also shows typical conditions under which the food enzyme is used.

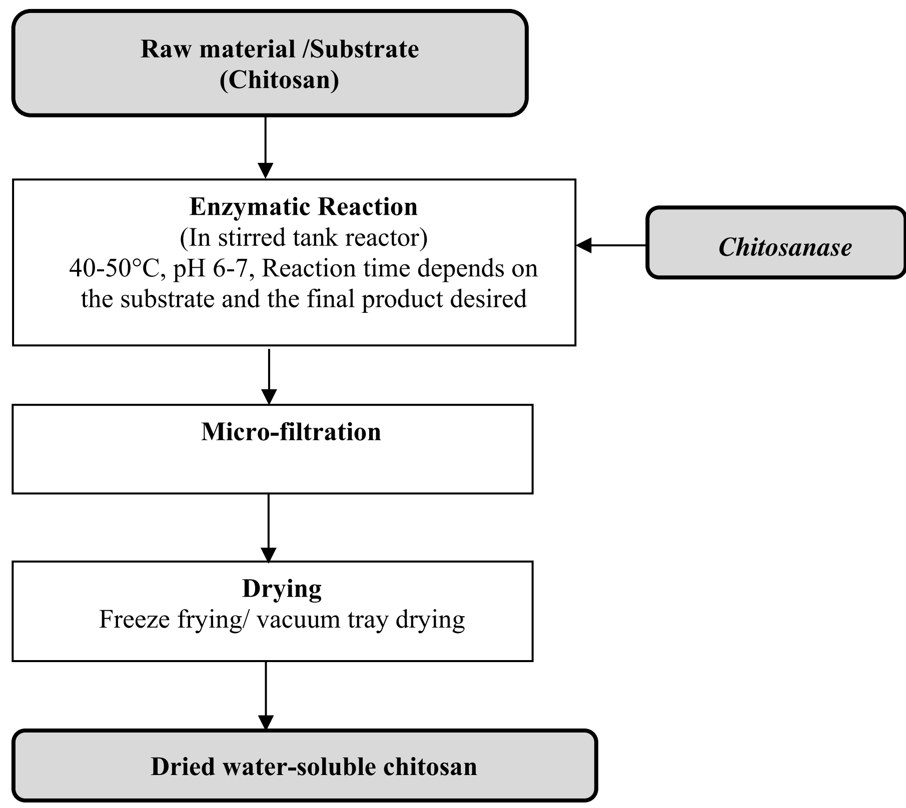



**Fig. 8: Flow-chart – Preparation of enzymatically hydrolyzed water-soluble chitosan using Chitosanase**

In general, the individual food processors see to it that the pH- and temperature conditions are such that the food enzyme can perform its technological function during food processing.

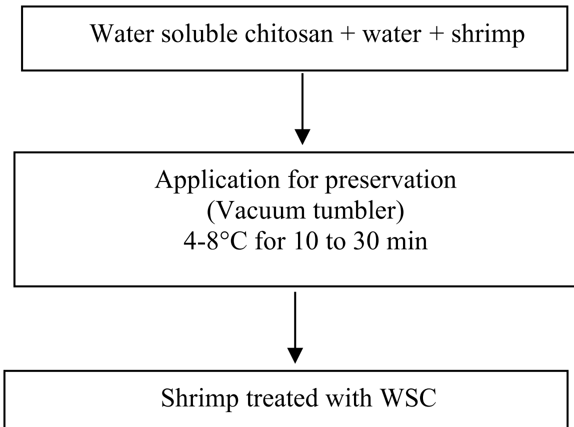



**Fig 9: Flow-chart - Use of water-soluble chitosan in the preparation of frozen shrimps**

Additionally, Chitosanase enzymes have been shown to be effective against fungal (yeast/mold) pathogens. Chitosanases are generally believed to play an important defense role against invading pathogens because of their potential to hydrolyze fungal cell wall polysaccharides (Ouakfaoui et. al. 1992). Low and high molecular weight chitooligosaccharides perform potent antimicrobial, antitumor and immunopotentiating functions (Matheson D et al., 1984).

### *2.5.3 USE LEVELS*

The Chitosanase described herein is intended to be used as a processing aid in the production of enzymatically hydrolyzed Water-Soluble Chitosan (WSC), which is subsequently to be used as a preservative in food industry, more specifically in shrimp preservation at usage rates consistent with current Good Manufacturing Practice

Studies conducted in house suggest that a maximum 3 g (i.e. 2.55 g TOS/kg) of Chitosanase of at least 2000U/g enzyme activity is required to convert one kg of chitosan to enzymatically hydrolyzed WSC (i.e. enzyme dose 3000 ppm)[9]. A review of literature (Chouljenko et al. 2016) and available data suggest that the shelf life of shrimp can be enhanced by applying approximately 5g water-soluble chitosan per kg of shrimp. Considering raw material to finished product ratio is 1 and total enzyme quantity used in the reaction, ends up in WSC, then the maximum quantity of the Chitosanase in the WSC will not be more than 15 ppm[10] or 12.74 mg TOS/kg [11].

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[9] Considering, maximum 3 g chitosanase used for hydrolysis 1kg of Chitosan i.e. 3000mg/kg OR 3000 ppm

[10] 5g/L of water-soluble chitosan is applied to 1 kg shrimp for the preparation of frozen shrimp to extend the shelf life and quality. Therefore (5/1000 x 3) = 0.015 g/kg i.e. 15mg/kg OR 15 ppm chitosanase is present in the 1 kg frozen shrimp

[11] TOS of chitosanase is estimated as 84.92%

Total organic solids (TOS) is the sum of all organic compounds present in the enzyme preparation derived from the enzyme source and manufacturing process. TOS is calculated as follows: TOS (%) = 100 – (A + W + D, where: A = % ash, W = % water, D = % diluents and other formulation ingredients.

### *2.5.4 ACTIVE AND INACTIVE ENZYME RESIDUES IN THE FINAL FOOD*

The fate of the Chitosanase used in the preparation of enzymatically hydrolysed WSC is described below.

In a stirred tank reactor, chitosan is mixed uniformly with appropriate buffer at controlled pH and temperature. Chitosanase is added to the reaction mixture and reaction continued till all the chitosan is solubilized. This reaction mixture can be purified further by microfiltration membrane to separate any insoluble impurities present in it. The filtrate is then dried and +water-soluble chitosan is obtained.

The presence of the enzyme Chitosanase in final food (WSC) is inconsequential, as the substrate is depleted thus the enzyme does not exhibit any technological effect in the final food.

As the enzyme Chitosanase is not physically removed from the process, it is assumed that the enzyme used in the reaction is present in the WSC. To perform a risk assessment, it is assumed that all the Chitosanase used in the reaction to produce WSC ends up in the final food (i.e., shrimp) and consumed by humans.

## 2.6 ALLERGENICITY

Allergenic potential of the Chitosanase was assessed following the pepsin digestion of the Chitosanase and bioinformatics analyses of its amino acid sequence.

### *2.6.1 EVALUATION OF ALLERGENICITY OF THE CHITOSANASE FOLLOWING PEPSIN DIGESTION METHOD*

Proteins that are susceptible to digestion by pepsin are less likely to elicit an allergenic response. Hence the digestibility of the Chitosanase by pepsin, was evaluated in vitro by exposing it to the action of pepsin in the presence of simulated gastric fluid (SGF).

Pepsin digestion study is based on the recommendations made by the Joint FAO/WHO Expert Consultation (2001). The protocol used for this assay is based on the detailed study undertaken by Thomas et *al*. (2004), which in turn takes into consideration the guidelines provided by the Codex Alimentarius Commission, (2003).

Results from this study show that Chitosanase can be considered to be a labile protein, as it is digested by pepsin in 30 seconds, and therefore is not likely to be allergenic.

### *2.6.2 SEQUENCE HOMOLOGY OF THE CHITOSANASE PROTEIN WITH THE KNOWN ALLERGENS*

Over the last decade, bioinformatics methods have been widely used for collecting, storing, and analyzing molecular and/or clinical information related to allergenicity of proteins. In order to address this point, information obtained from bioinformatics, coupled with experimental data, wherever necessary, is the approach postulated by the joint Food and Agriculture Organization and World Health Organization (FAO/WHO) Expert Consultation on Allergenicity of Foods Derived from Biotechnology. This approach takes into account the evidence derived from several types of information and data since no single criterion is sufficiently predictive (Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology).

As proposed in the FAO/WHO Report, cross-reactivity between a query protein and a known allergen has to be considered when there is: (a) more than 35% identity in the amino acid sequence of the test protein, using a window of 80 amino acids and a suitable gap penalty, or (b) A stretch identity of 6-8 contiguous amino acids between the test protein and known allergen.

The Chitosanase sequence (as discussed in the section 2.1.2) was analyzed using the SDAP online tool to determine homology to known allergens.[12] Bioinformatics searches showed that Chitosanase from *B. subtilis* CSSC shares no homology to known allergens over a sliding window of 80 amino acids or full length. Additionally, no hits were obtained for a match of 8 contiguous amino acids.

When assessing the risk of allergenicity in proteins it is important to note that a very small quantities of the food enzyme(s) are used during food processing, resulting in miniscule quantities of the enzyme(s) in the final food. Goodman et al (2008) discuss that a high concentration generally equals a higher risk of sensitization, whereas a low level in the final food equals a lower risk. Additionally, it must be noted that the food enzyme protein undergoes denaturation under various conditions of food processing, resulting in loss of its tertiary structure. In general, these alterations in conformation are associated with decrease in the antigenic reactivity in humans. Usually, denatured proteins are much less immunogenic than the corresponding native proteins [Takai et al., 1997; Takai et al., 2000 (as cited in Koyanagi et al. 2010); Valenta, 2002; Kikuchi et al., 2006]. Additionally, residual enzyme(s) still present in the final food will be subjected to digestion in the gastro-intestinal system (as demonstrated in the gastric digestion model, See section 2.6.1), which further reduces the risk of enzyme allergenicity. It is believed that small protein fragments resulting from digestion are less likely to be allergenic (FAO/WHO, 2001; Goodman et al., 2008).

The results presented above suggest that the Chitosanase enzyme is readily digested by the pepsin making it unlikely that the protein is allergenic in humans. In addition, bioinformatics analyses indicate that there is no similarity of the Chitosanase amino acid sequence with known allergen amino acid sequences. These observations, when coupled with the very low exposure to consumers by the processing aid use of the Chitosanase, indicate that the risk of allergenicity elicited by Chitosanase enzyme protein is very low.

### *2.6.3 ALLERGENICITY OF ENZYME PREPARATIONS*

Fermentation medium used for production of the Chitosanase contains soya protein, which is one of the eight known allergens listed in FALCPA (2004).[13] Use of soy flour as a nitrogen source is very common in the industrial bacterial fermentations. According to the Enzyme Technical Association position on the labelling of enzymes, enzymes do not fall under the eight major allergic foods and hence do not fit within the first labelling requirement of Food Allergy Labeling and Consumer Protection Act (FALCPA, 2004).[14]

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[12] SDAP - Structural Database of Allergenic Proteins (https://fermi.utmb.edu/SDAP/sdap_who.html)
[13] Food Allergen and Consumer Protection Act, 2004
[14] 'Food Allergen Labeling of Microbially Derived Enzymes' Under FALCPA as it applies to Fermentation Media Raw Materials' (2004), https://www.enzymetechnicalassociation.org/wp-content/uploads/2018/10/Allergen-psn-paper-2.pdf; last accessed September 28, 2020.

Additionally, the enzyme preparations are not derived from the major food allergens nor are the enzymes a byproduct of the major food allergens. The fermentation media in general provide a limited amount of nitrogen source which is consumed during growth of the microorganism. In effect, the soy protein is a nutrient and not an ingredient in the enzyme preparation. We note, further, that downstream processing of the enzyme preparation is designed to remove unhydrolyzed raw materials. Thus, the Chitosanase enzyme preparation does not fall under the labelling requirements outlined in FALCPA (2004)

## 2.7 Oral Toxicity and Genotoxicity Studies

Chitosanase from *B. subtilis* CSSC, the notified enzyme preparation, has been investigated in a series of oral and genotoxicity studies, which comply with OECD Guidelines and conducted in accordance with the principles of Good Laboratory Practice (GLP)[15]

### *2.7.1 ACUTE ORAL TOXICITY TEST (OECD TEST NO. 423, 2001)*

Using the step-wise method, 2 groups of n=3 female Sprague Dawley rats aged 8-12 weeks and weighing 196.1-208.0 g were dosed via gavage with 2000 mg TOS preparation (5750 U)/kg bw and observed for 14 days. No indications of toxicity were reported. Based on the results, the estimated LD50 for Chitosanase from *B. subtilis* CSSC was greater than 2000 mg TOS/kg bw. (Annex A1)

### *2.7.2 REPEATED-DOSE 90-DAY ORAL TOXICITY TEST (OECD TEST NO. 408, 1998)*

Four groups of 10 male and 10 female Wistar rats, 5-7 weeks old and weighing 110.9-142.9 g (males, mean = 123.40 g) and 110.5-133.8 g (females, mean = 120.11 g) were assigned to receive daily oral gavage of doses of 0, 250, 500, and 1000 mg TOS/kg bw (providing 0, 718.75, 1437.5, and 2875 U/kg bw) for 90 days. Groups of 5 rats/sex receiving 0 or 1000 mg TOS of enzyme preparation/kg bw/day were assigned to 28-day recovery groups. Rats were examined daily for signs of toxicity, morbidity, and mortality. Test animals were subjected to detailed clinical examinations at day 0 and weekly thereafter during the treatment and recovery period. Ophthalmic examinations were performed on the control and high-dose rats at beginning and end of dosing. At week 13, all animals were assessed for sensory reactivity, grip strength, and motor activity. Feed consumption and body weight were recorded weekly. Blood and urine samples were taken at the end of dosing and after recovery. All animals were subjected to necropsy and weights of kidneys, liver, adrenals, testes, epididymis, uterus, thymus, spleen, brain, ovaries, and heart were recorded. Histological evaluations were performed on all tissues from control and high-dose rats.

There was no mortality and no clinical abnormalities observed in rats treated at any dose. Ophthalmological examination revealed no abnormalities, nor did the neurotoxic assessment. There was no effect on feed intake or body weight gain, hematological or biochemical parameters, absolute or relative organ weights and no histopathology. The No Observed Adverse Effect level (NOAEL) of Chitosanase from *B. subtilis* CSSC in the Sprague Dawley

[15] Organization for Economic Co-Operation and Development, OECD (ENV/MC/CHEM (98)17)

rat, following oral administration for 90 days, was the highest dose tested, 1000 mg TOS/kg bw/day providing 2875 U/kg bw/day. (Annex A2)

### *2.7.3 BACTERIAL REVERSE MUTATION TEST—AMES ASSAY (OECD TEST NO. 471, 1997)*

The test was conducted using *Salmonella typhimurium* test strains TA97a, TA98, TA100, TA102, and TA1535 in the presence and absence of S9 metabolic activation. The test was conducted in triplicate at concentrations of 0, 61.72, 185.18, 555.55, 1666.66, and 5000 µg TOS/plate. No significant increase in the number of histidine revertant colonies was reported, and it is concluded that, under the conditions of this study, Chitosanase preparation from *B. subtilis* CSSC is non-mutagenic. (Annex A3)

### *2.7.4 IN VITRO MAMMALIAN CHROMOSOMAL ABERRATION TEST IN HUMAN LYMPHOCYTES (OECD TEST NO. 473, 2014)*

Cultures of human peripheral blood lymphocytes were exposed to the Chitosanase preparation from *B. subtilis* CSSC at concentrations of 0, 0.31, 0.62, 1.25, 2.5 and 5 mg TOS/ml in the presence and absence of metabolic activation system for 3 or 24 hours. No significant concentration related increase was reported in the incidence of structural chromosome aberrations at any tested concentration, and it was concluded that Chitosanase enzyme preparation from *B. subtilis* CSSC is non-clastogenic in the presence and absence of microsomal enzymes. (Annex A4)

# Part 3: 21 CFR 170.235: Intended Use and Dietary Exposure

## 3.1 Intended Use and Dietary Exposure

The Chitosanase preparation described in this Notice is intended to be used as a processing aid for production of enzymatically hydrolyzed Water-Soluble Chitosan (WSC). WSC is to be used in preserving shrimp, which are consumed by the general population.

To estimate the human consumption of the food enzyme Chitosanase preparation, the average seafood consumption is considered in the populations across United State of America, and quantity of the enzyme present is the amount of seafood consumed is calculated. In this calculation it is assumed that the entire Chitosanase preparation used in the production of WSC is carried over in the final product, which is then consumed by the population. In addition, it is assumed that all of the seafood consumed in the US is consumed in the form of shrimp.

In-house studies suggest that approximately 3 g of Chitosanase (2000U/g enzyme activity) is required to convert one kg of chitosan to WSC (i.e. enzyme dose is 3000 ppm, raw material to finished product is approximately 1)[16]. This dose, however, needs to be adjusted according to the quality of input raw materials, as the starting raw material- chitosan may vary in composition depending on source and processing conditions. A review of literature (Chouljenko et al. 2016) and available information suggest use of 5 g WSC per kg of shrimps for preservation purposes. Assuming that the whole amount of enzyme used in reaction ends up in the product, i.e., WSC, the maximum possible quantity of the Chitosanase preparation in the final shrimp product will not be more than 15 ppm[17] or12.74 mg TOS/kg[18].

In order to estimate the probable human exposure to Chitosanase on a continual daily basis, Advanced Enzymes has relied upon (1) potential shrimp-derived chitosan use from recent published studies (Chouljenko et al. 2016, Yu Guang-li et al. 1996), and (2) the results of its own food technology research. According to the data available from United State Department of Agriculture Economic Research Service, the average daily seafood consumption by US population is 19.26 g (15.5 pounds/person/year) (USDA, Seafood consumption data, 2016; National Marine Fisheries Service United States, 2017; American seafood consumption, 2015) Based upon the aforementioned data and assuming that all seafood consumed is consumed in the form of shrimp, the estimated daily intake (EDI) of Chitosanase preparation is 0.245 mg TOS/person/day[19]. Considering the average weight of male adult to be 70 kg, the Estimated Dietary Intake (EDI) of the Chitosanase preparation is 0.0035 mg TOS/kg body weight.[20]

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[16] Assuming 3 g chitosanase is used for the hydrolysis of 1kg of Chitosan i.e. 3000mg/kg OR 3000 ppm

[17] 1 Liter of 5g/L water-soluble chitosan is applied to 1 kg shrimp for the preparation of frozen shrimp to extend the shelf life and quality. Therefore (5/1000 x 3) = 0.015 g Chitosanase preparation/kg of shrimp i.e. 15mg/kg OR 15 ppm Chitosanase is present in the 1 kg frozen shrimp

[18] TOS of Chitosanase is estimated as 84.92%

Total organic solids (TOS) is the sum of all organic compounds present in the enzyme preparation derived from the enzyme source and manufacturing process. TOS is calculated as follows:

TOS (%) = 100 – (A + W + D), where: A = % ash, W = % water, D = % diluents and other formulation ingredients.

[19] Estimated Daily Intake :19.26 g of raw material (frozen shrimp) contains (12.74 x 19.26/1000) = 0.245mg TOS/person/day of Chitosanase preparation.

[20] 0.245/70 kg body weight = 0.0035 mg TOS/kg body weight/day of Chitosanase.

The NOAEL dose for the Chitosanase preparation from *Bacillus subtilis* CSSC, concluded from the 90-day toxicity study is 1000 mg TOS/kg bw/day (Annex A2), corresponding to 2875U/kg body weight or 70g TOS/person/day, assuming an average 70kg male consumer.

The maximal Acceptable Daily Intake (ADI) concluded from the NOAEL dose is the NOAEL dose adjusted for a 100x safety factor and represents the highest level of consumption that is considered safe.[21] The ADI for Chitosanase enzyme preparation used as a processing aid in the production of WSC is 700 mg TOS/person/day or 2013 U/person/day.[22] The EDI for Chitosanase resulting from the intended use is 0.245 mg TOS/person/day and well below the ADI determined by test.

We note, specifically, that the assumptions used in the calculations for EDI and ADI (listed below) are highly conservative and represent "worst case" consumption estimates that are highly unlikely to occur.

- All seafood consumed in the US is consumed in the form of shrimp
- All seafood consumed as shrimp in the US will be treated with WSC
- Shrimp will be treated with WSC at the maximal levels used in the Chitosanase enzyme EDI calculation and all the WSC will remain in the shrimp
- Chitosanase enzyme preparation will be used at the maximal usage rate for the conversion of chitin to WSC

## 3.2 Margin of Safety

While it is clear that the calculated EDI (0.245 mg TOS/person/day) is well below the ADI( 700 mg TOS/person/day) derived from the 90-day toxicology study[23], indicating that the use of Chitosanase as a processing aid in the production WSC is safe for consumption at the intended usage levels, a quantitative measure of the degree of safety can be determined as a Margin of Safety (MOS). MoS for human consumption of a food can be calculated by dividing the NOAEL (70000 mg TOS/person/day), derived by the 90-day toxicology tests as described above, by the EDI value of 0.245 mg TOS/person/day.

**Margin of Safety (MoS) = 70000 mg TOS/person/day/0.245 mg TOS/person/day = 285714**

The margin of safety is calculated assuming that all the enzyme used in chitosan processing ends up in the product, i.e., WSC. The safety margin is extremely high and provides a strong indication that the use of the Chitosanase preparation to produce WSC poses no safety concern for consumers. For perspective, a MoS equal to one (1) would be acceptable to establish the safety of an ingredient in food.

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[21] 21 CFR 170.22

[22] ADI = NOAEL x 70/100, where body weight of a healthy individual is considered 70 kg and a safety factor of 100 is considered for determination of a safe dose for healthy human adults and children.

[23] Ibid

# Part 4: 21 CFR 170.240: Self-Limiting Levels of Use

There are no self-limiting levels of use of Chitosanase from *B. subtilis* CSSC in food applications.

# Part 5: 21 CFR 170.245: Experience Based on Common Use in Food before 1958

While Chitosanase has a history of use in food as discussed herein, the statutory basis for our conclusion of GRAS status in this Notice is scientific procedures rather than on common use in food prior to 1958.

# Part 6: 21 CFR 170.250: Narrative

## 6.1. Safety of the Production Strain

*B. subtilis* CSSC is the source organism (production strain) that is used for the production of Chitosanase described in this Notice.

*Bacillus subtilis* is among the most widely used bacteria for the industrial scale production of enzymes. It is considered as a safe production organism for enzymes used in food / feed processing as well as for numerous other industrial applications. Secondary metabolites show no safety concern in fermentation products derived from *Bacillus subtilis*. Thus, *Bacillus subtilis* can be considered generally safe, not only as a production organism of its natural enzymes, but also as a harmless host for other gene products.

- *Bacillus subtilis* is not considered to be a pathogen and is generally represented as an example of non-pathogenic organisms (FDA, 1986).
- The US EPA added *Bacillus subtilis* to the list of exempted organisms in 1997 (US EPA, 1997).
- The American Type Culture Collection (ATCC, 2020) classifies different strains of *B. subtilis* as Bio-safety Level 1, indicating that it is a well-characterized agent which does not cause disease in healthy humans.
- In 1999, FDA affirmed that carbohydrase enzyme preparation from either *Bacillus subtilis* or *Bacillus amyloliquefaciens* are considered as generally recognized as safe (GRAS) for use as direct food ingredients (21 CFR part 184.1148, 1999).
- There are at least nine FDA GRNs that provided for the use of *B. subtilis* as a production host microorganism, all of which have received a positive "FDA has no questions" letter (GRN 649, 592, 579, 476, 406, 274, 205, 114, and 20).
- EFSA has assigned *Bacillus subtilis* a "Status of Qualified Presumption of Safety" [QPS] (EFSA 2007, 2018). Further, B. *subtilis* does not appear on the list of pathogens in Annex III of Directive 2000/54/EC, as it is globally regarded as a safe microorganism.
- Numerous enzyme preparations from *Bacillus subtilis* have undergone safety evaluations by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), including *Bacillus subtilis* sources of xylanase, amylase, mixed microbial carbohydrase and protease (JECFA, 2004, 1990, 1971).
- *Bacillus subtilis* is considered safe as a viable microbial ingredient for human oral consumption (Hong et al., 2008; Sorokulova et al., 2008). Use of *Bacillus subtilis* as food ingredient in commercial food preparations is described in GRN 000831. *B. subtilis* strains are being used as starter culture for fermentation of various foods, fodders and feed additives for centuries (Wang & Fung, 1996; Harwood et al., 2018). It is being used in the preparation of health promoting traditional fermented foods such as natto (Southeast Asia) and ogiri (Africa), dawadwa (West Africa), Axone and Piak (India), shuǐdòuchǐ càixīn in (China), Thuanao (Thailand), Cheonggukjang (Korea) (Lefevre et al., 2016; Cutting, 2011; Arora et al., 1992; Shieh, 2009). *B. subtilis* is a dominant microflora of fermented maize product pozol (Wacher et al., 1993).

Chtiosanase produced by *B. subtilis* CSSC, had shown no safety concern when evaluated for acute oral toxicity (OECD 423), repeated dose 90 day oral toxicity test (OECD 408), bacterial reverse mutation test (OECD 471) and *In vitro* mammalian chromosomal aberration test (OECD

473) (Section 6.4), confirming safety of the production strain as well. In addition, *B. subtilis* CSSC was found not to be cytotoxic in a Vero cell assay and does not produce endotoxins that are known to be produced by other species in the *Bacillus* genus.

The information presented above clearly establishes safety of the production strain *B. subtilis* CSSC for the production of Chitosanase for use as a processing aid in the production of WSC used for shrimp preservation.

## 6.2 Chitosanase Use and Dietary Exposure

The Chitosanase enzyme preparation described herein is intended to be used as a processing aid in the production of enzymatically hydrolyzed WSC, which is subsequently to be used as a preservative in food industry, more specifically in shrimp preservation at usage rates consistent with current Good Manufacturing Practice.

The dietary exposure to the Chitosanase enzyme preparation resulting from consumption of shrimp treated with WSC is very low consistent with the use of the enzyme as a processing aid in the production of WSC that is subsequently used as an ingredient in shrimp processing. Using highly conservative assumptions of shrimp intake, WSC usage rates in processing shrimp and usage rates of Chitosanase enzyme in processing WSC, Advanced Enzymes calculates the EDI of Chitosanase preparation resulting from eating shrimp would be 0.245 mg TOS/person/day.

## 6.3 Safety of the Chitosanase Preparation

Chitosanase has a history of use in the food industry. Chitosanase produced from the *Bacillus* genus is listed as food additives by Korea Food and Drug Administration. It is also recognized as food additive from natural origin by the Ministry of Health and Welfare Japan on April 16, 1996. Health Canada has classified Chitosanase as a Natural Health Product under schedule 1 and has also approved as per China Food Additive regulation amendment published in 2018 (NHC, 11th Amendment for GB 2760-2014).

Chitosanase enzymes are ubiquitous in nature. They are present in microorganisms, and also reported in plants consumed by humans (Somashekhar et al. 1996). In the human body, chitosan gets degraded due to the activity of lysozyme and bacterial enzymes present in the colon. The human colonic microbiome is also known to produce chitosan-degrading enzymes (Loncarevic et al. 2017). This implies that Chitosanase can be naturally present in human diet and potentially present in the human GI tract as well. Based on simulated gastric digestion tests, the enzyme protein can be digested by gut proteases like any other protein in the human body. Hence the addition of Chitosanase in the proposed application is unlikely to have a significant effect on the human body.

Further, the safety of Chitosanase preparation from *B. subtilis* CSSC was evaluated in animal and other cell-based models. Studies carried out include acute, sub chronic studies of oral toxicity and in vitro genetic toxicity assays. The Chitosanase was found to be non-toxic, non-mutagenic and non-clastogenic under the given test conditions at the highest dose tested. (Annexes A1-A4).

The NOAEL dose for the Chitosanase preparation from *Bacillus subtilis* CSSC, concluded from the 90-day toxicity study is 1000 mg TOS/kg bw/day or 70g TOS/person/day, assuming an average 70kg male consumer (Annex 2A).

The maximal Acceptable Daily Intake (ADI) calculated from the NOAEL dose is the NOAEL dose adjusted for a 100x safety factor and represents the highest level of consumption that is considered safe. The ADI for Chitosanase enzyme preparation used as a processing aid in the production of WSC, is 700 mg TOS/person/day. The EDI for Chitosanase resulting from the intended use is 0.245 mg TOS/person/day and well below the ADI determined by test with a MoS (NOAEL/EDI) of 285714.

The safety evaluation of Chitosanase use as a processing aid in the manufacture of WSC included evaluating the potential of the enzyme to elicit an allergenic response. Digestibility of a protein in a gastric digestion simulation mode is considered a model for allergenicity potential. Chitosanase from *B. subtilis* CSSC was completely digested in the model system with pepsin within 30 minutes indicating a low potential for allergenicity. Further, the amino acid sequence of the enzyme was compared to known allergen sequences and had no homology to these proteins. The results of these two methods coupled with the very low exposure levels from the intended use strongly indicate that there is a low risk of allergenicity in the intended use.

Finally from a human gut ecology perspective, Chitosanase from *B. subtilis* CSSC was evaluated for its antimicrobial activity following FAO/JECFA (2006) and EFSA (2010), against six selected microorganisms [*Staphylococcus aureus* (ATCC 6538); *Escherichia coli* (ATCC 11229); *Bacillus cereus* (ATCC 2); *Bacillus circulans* (ATCC 4516); *Streptococcus pyrogenes* (ATCC 12344) and *Serratia marcescens* (ATCC 14041)]. Chitosanase showed an absence of antimicrobial activity against the selected test micro-organisms and is unlikely to have a negative impact on the gut microflora.

## 6.4 Regulatory History and Leading Enzyme Publications on Safety of the Enzyme

Chitosanase is known to be consumed safely by the general human population. There is precedence of regulatory recognition of Chitosanase. Following summarizes regulatory recognition for Chitosanase.

- Chitosanase from *Bacillus* genus is specified as food additives by Korea Food and Drug Administration.
- Chitosanase is recognized as food additive from natural origin by the Ministry of Health and Welfare Japan on April 16, 1996.
- Health Canada has classified Chitosanase as a Natural Health Product under schedule 1.
- National Health Commission (NHC) of China has approved Chitosanase from *Bacillus subtilis* as a food additive as per Food Additive regulation (GB 2760-2014) amendment published in 2018.

Chitosanase produced from *B. subtilis* can be used as a biocatalyst for industrial scale production chitosan oligosaccharides. Chitosan oligosaccharides (COSs) are produced by hydrolysis of chitosan (the deacetylation product of chitin) by Chitosanases. COSs are widely used in food, textile, pharmaceutical, and medical applications. (Su et al 2017, Pechsrichuang P et al, 2013)

Studies on Chitosanase produced by *B. subtilis* conducted by Su et al. (2017) showed a promising ability of this enzyme to hydrolyze chitosan to $(GlcNAc)_2$, $(GlcNAc)_3$ and $(GlcNAc)_4$.

## 6.5 Safety of Manufacturing Process

The Chitosanase described in this dossier is manufactured in accordance with current Good Manufacturing Practice (cGMP) and the principles of Hazard Analysis and Critical Control Points (HACCP) as described in 21CFR§110 and 117. The manufacturing facility is ISO 9001, ISO 22000 and GMP certified.

## 6.6 Decision Tree

The decision tree proposed by Pariza and Johnson (2001) was used to aid in the determination of safety for the processing aid use of Chitosanase from *B. subtilis* CSSC in food at usage rates in accordance with cGMP. The results of the decision tree are indicated in Figure 10 below, where the Chitosanase was determined to be acceptable for use in food processing.

**Figure 10. Decision tree determination of safety of Chitosanase from *B. subtilis* CSSC**

The safety of Chitosanase from *B. subtilis* CSSC has also been established using the decision tree for evaluating the safety of microbial enzyme preparations used in food processing (Pariza et al. 2001)

1. Is the production strain genetically modified? **NO**

2. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure? **NO**

3. Is the organism nonpathogenic? **YES**

4. Is the test article free of antibiotics? **YES**

5. Is the test article free of oral toxins known to be produced by other members of the same species? **YES**

6. Is the NOAEL for the test article in appropriate oral studies sufficiently high to ensure safety? **YES, the test article is ACCEPTED***

## 6.7 GRAS Determination

Advanced Enzymes employed scientific procedures in determining the safety of Chitosanase preparation from *B. subtilus* CSSC. These procedures include:

- Establishing the safety of the production organism.
- Identifying and characterizing the Chitosanase preparation.
- Appropriate toxicological testing of the Chitosanase preparation providing NOAEL and ADI values.
- Estimation of dietary intake from the intended use based upon recognized consumption statistics and product testing to establish cGMP levels of use.
- Determination of allergenicity potential via gastric digestion simulation and bioinformatic analysis of the enzyme amino acid sequence.
- Setting appropriate specifications and testing protocols.
- Demonstrating by test that the manufacturing process is robust and capable of producing product that meets specification.
- Appropriate literature survey to establish a safe history of use in food of the enzyme and production organism, including relevant regulations.

In summary, *Bacillus subtilis* is widely used by enzyme manufacturers around the world to produce enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. Further, it is also used as direct food ingredient in commercial food preparations *Bacillus subtilis* is a considered safe host for enzyme production as well as it is considered non-toxigenic, non-pathogenic organism. As is the case for the production organism, Chitosanase has a safe history of use in food and is approved for food use in a number of jurisdictions

In addition to the widely regarded safety of the production organism and the safe history of use of the enzyme in food, the multi-phasic safety evaluation of Chitosanase from *B. subtilis* CSSC described in this Notice overwhelmingly indicates that the usage of the enzyme as a processing aid for the production WSC to be used in shrimp processing at levels consistent with cGMP is reasonably certain to be safe.

The enzyme is well characterized and unambiguously identified as a Chitosanase preparation and Chitosanase enzymes have a safe history of use in food. Chitosanase preparation testing indicates that the enzyme is readily and rapidly digested by pepsin and has no amino acid sequence homology to known allergens, indicating that the enzyme preparation presents very low risk of allergenicity. Toxicology testing did not reveal any adverse effects of Chitosanase treatment at the highest dose of 1000mg/animal/day. The NOAEL based upon the highest dose tested was established and an ADI of 700mg TOS/person/day was calculated. When comparing the ADI with a highly conservative "worst case" scenario EDI, a Margin of Safety of 2,854 was established.

The Chitosanase *B. subtilus* CSSC is manufactured in accordance with current Good Manufacturing Practice for Food (cGMP) and the principles of Hazard Analysis of Critical Control Points (HACCP) in compliance with 21CFR110 and 117.

Advanced Enzymes has determined, employing the Pariza and Johnson (2001) decision tree, based upon the information obtained by scientific procedures, that the safety of the production organism, the safe history of use in food of Chitosanase preparation in food, the safety of the Chitosanase preparation for the intended use as established by extensive testing, and the utilization of appropriate manufacturing for the production of food overwhelmingly support the conclusion that Chitosanase from *B. subtilis* CSSC is Generally Regarded as Safe for use as a processing aid in the manufacture of WSC at levels in accord with cGMP.

# Part 7: 21 CFR 170.255: List of supporting data and information

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26-FEB-2015
IIT STUDY NUMBER 18102

# INDIAN INSTITUTE OF TOXICOLOGY

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## FINAL REPORT
## IIT STUDY NUMBER 18102
## ACUTE ORAL TOXICITY OF CHITOSANASE IN RAT

**STUDY DIRECTOR**
**Dr. R.M.Bhide Ph.D., ERT**

**TESTING FACILITY :**
**INDIAN INSTITUTE OF TOXICOLOGY**
**32 A/1, Hadapsar Industrial Estate,**
**Pune - 411 013.**

**SPONSOR'S REPRESENTATIVE**
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**SPONSOR ADDRESS :**
**ADVANCED ENZYME TECHNOLOGIES LTD.,**
**Sun Magnetica, 'A' wing, 5th Floor,**
**LIC Service Road,**
**Louiswadi, Thane (W) 400 604**
**Maharashtra, India.**

**REGULATORY REQUIREMENTS:**

OECD guideline for the Testing of Chemicals (No. 423, Section 4: Health Effects) "Acute Oral Toxicity - Acute Toxic Class Method "Adopted on 17th December 2001.

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## STATEMENT OF GLP COMPLIANCE

| | |
|---|---|
| Title of the Study | : Acute Oral Toxicity of Chitosanase in Rat |
| Study Number | : 18102 |
| Study Plan Number | : SPL/002/059 |
| Name of the Test item | : Chitosanase |

The study was conducted in accordance with the Good Laboratory Practice Principles as Published by the OECD in 1998, No 1 ENV/MC/CHEM(98)17.

| Mr. V.M.Bhide M.B.A. | | 26.02.2015 |
|---|---|---|
| Test Facility Management | Signature | Date |

## STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE

| | |
|---|---|
| Title of the Study | : Acute Oral Toxicity of Chitosanase in Rat |
| Study Number | : 18102 |
| Study Plan Number | : SPL/002/059 |
| Name of the Test item | : Chitosanase |

The study was conducted in compliance to the Good Laboratory Practice Principles as Published by OECD in 1998, No 1 ENV/MC/CHEM(98)17.

The study was conducted in compliance to the written Study Plan approved by the Study Director and authorized by the Sponsor and Indian Institute of Toxicology Management and all applicable Standard Operating Procedures of Indian Institute of Toxicology, Pune.

All original Raw Data including documentation, signed Study Plan, Study Plan Amendments and a copy of Final Report are archived at Indian Institute of Toxicology, Pune.

I accept responsibility for the conduct of the study and hereby declare that the study was conducted under my direction. This report is a complete, true and accurate record of the results obtained.

The sponsor is responsible for Good laboratory Practice (GLP) compliance for all Test Item information unless determined by the Indian Institute of Toxicology.

| Dr. R.M.Bhide Ph.D., ERT | | 26/2/2015 |
|---|---|---|
| Study Director | Signature | Date |

## STATEMENT OF QUALITY ASSURANCE UNIT

Title of the Study : Acute Oral Toxicity of Chitosanase in Rat

Study Number : 18102

Study Plan Number : SPL/002/059

Name of the Test item : Chitosanase

This study has been audited and the final report has been examined with respect to study plan, SOP and raw data. The report is true reflection of the raw data and the study was conducted in compliance with the principles of GLP. The audits were carried out according to the applicable SOP's of Quality Assurance Unit of Indian Institute of Toxicology, Pune. The report is kept in the archives at Indian Institute of Toxicology, Pune.

Inspections were made by the Quality Assurance Unit of the Indian Institute of Toxicology for different phases of the study described in this report. The dates on which the inspections were made and the dates on which the findings were reported to the Study Director and to the facility Management are given below.

| Date(s) of Inspection | Phases Inspected | Date(s) findings reported to Study Director | Date(s) findings reported to Management |
|---|---|---|---|
| 16/12/2014 | Study Plan Review | 16/12/2014 | 16/12/2014 |
| 26/12/2014 | Pre-study Verification | 26/12/2014 | 26/12/2014 |
| 26/12/2014 | Amendment - 1 | 26/12/2014 | 26/12/2014 |
| 01/01/2015 | Test Item Administration | 01/01/2015 | 01/01/2015 |
| 08/01/2015 | Body Weights | 08/01/2015 | 08/01/2015 |
| 17/02/2015 | Raw Data Audit | 17/02/2015 | 17/02/2015 |
| 17/02/2015 | Draft Report Audit | 17/02/2015 | 17/02/2015 |
| 25/02/2015 | Amendment - 2 | 25/02/2015 | 25/02/2015 |
| 26/02/2015 | Final Report Audit | 26/02/2015 | 26/02/2015 |

| Dr. P.R.Tikhe Ph.D. | | 26.02.2015 |
|---|---|---|
| Quality Assurance Unit | Signature | Date |

# PERSONNEL INVOLVED IN THE STUDY

| | |
|---|---|
| Study Director | : **Dr. R.M.Bhide** Ph.D., ERT |
| Veterinarian | : **Dr. S.N.Khutale** M.V.Sc. |
| Study Scientists | : **Miss S.V.Patil** M.Sc.<br>**Mr. D.D.Gawande** M.Pharm.<br>**Mr. M.P.Pawar** B.Sc.<br>**Mr. M.P.Supekar** M.Pharm. |
| Pathology | : **Dr. V.V.Dange** M.V.Sc.<br>**Dr. S.S.Kad** M.V.Sc. |
| Statistics | : **Mr. S.D.Nagpure** B.Com.<br>**Mr. D.K.Raut** H.S.C. |
| Quality Assurance Unit | : Dr. P.R.Tikhe Ph.D.<br>Dr. R.M.Gosavi M.V.Sc. |
| Report Preparation | : Dr. R.M.Bhide Ph.D., ERT<br>Mr. S.D.Nagpure B.Com. |

## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| IIT | - | Indian Institute of Toxicology |
| GLP | - | Good Laboratory Practice |
| OECD | - | Organization for Economic Co-operation and Development |
| SOP | - | Standard Operating Procedure |
| Mg | - | Milligram |
| G | - | Gram |
| kg | - | Kilogram |
| °C | - | Degree Celsius |
| % | - | Percent |
| No. | - | Number |
| Nos. | - | Numbers |
| ml | - | Milliliter |
| GHS | | Globally Harmonised System |
| e.g. | - | For Example |
| Ltd. | - | Limited |
| Contd. | - | Continued |
| TS | - | Terminal sacrifice |
| NAD | - | No abnormality detected |
| min. | - | Minutes |
| hrs. | - | Hours |
| TOS | - | Total Solid |

## SUMMARY AND CONCLUSION

The study now reported was designed and conducted to determine the acute oral toxicity profile of **Chitosanase** in Sprague Dawley rats.

Initially, three female animals were treated at the dose level of 2000 mg TOS/kg body weight of the test item. Administration of the test item at 2000 mg TOS/kg did not result in any signs of toxicity and mortality at 24 hours after the dosing, hence three female animals were added to the study and treated with the dose of 2000 mg TOS/kg of the test item.

No signs of toxicity or mortality were observed in all six female animals throughout the study period of 14 days when treated at the dose level of 2000 mg TOS/kg of the test item.

Gross pathological examination did not reveal any abnormalities in animals from 2000 mg TOS/kg dose group.

It was concluded that the acute toxicity study of **Chitosanase** supplied by **Advanced Enzyme Technologies Ltd., Thane,** when administered via oral route in Sprague Dawley rats falls into the category 5 criteria of Globally Harmonised System (GHS).

$LD_{50}$ cut off value: 5000 mg TOS/kg body weight. Category evaluation as per the Classification System (Annexure III).

**DR.R.M.BHIDE Ph.D., ERT**
**STUDY DIRECTOR**

1) The results relate only to the items tested.
2) This report shall not be reproduced except in full, without the written approval of the laboratory.

**Study Schedule**

| | |
|---|---|
| Study Initiation Date | : 16-12-2014 |
| Experimental Starting Date | : 31-12-2014 |
| Day of First Dosing | |
| Dose 2000 mg TOS/kg, Group I, Step I | : 01-01-2015 |
| Dose 2000 mg TOS/kg, Group I, Step II | : 02-01-2015 |
| Experimental Completion Date | : 17-01-2015 |
| Study Completion Date | : 26-02-2015 |
| Date of Reporting | : 26-02-2015 |

**Archives**

All original raw data, the signed study plan, study plan amendments and a copy of final report will be retained in the Archives at Indian Institute of Toxicology, Pune for a period of nine years. At the end of this period, the sponsor's instructions will be sought to either extend the archiving period or return the archived material to the sponsor or for the material to be disposed off.

**Test Item Return**

On completion of the study and submission of the final report, all unused samples of the test item will be returned to the Sponsor.

**Animal Welfare**

This study was performed as per the recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines for Laboratory Animal Facility.

**Purpose**

The purpose of this study was to assess the Toxicological profile of test item to a single administration via oral route to Sprague Dawley rats. This study was designed to determine the acute toxicity at fixed dose levels by oral route of the test item. This method provides information on the hazardous properties, and allows the test item to be ranked and classified according to Globally Harmonized System (GHS) for the classification and labeling of the test item.

# MATERIALS AND METHODS

## Test Item

| | |
|---|---|
| Sponsor | : **Advanced Enzyme Technologies Ltd., Thane** |
| Laboratory Sponsor Code | : **SPN/002** |
| Test Item | : **Chitosanase** |
| Batch Number | : **061434** |
| Laboratory Test Item Code | : **TAS/002/017** |
| Manufacturing Date | : **June, 2014** |
| Expiry Date | : **May, 2016** |
| Consistency | : **Solid powder** |
| Activity (Clinical Indication) | : **Biocatalyst for industry** |
| Safety Precautions | : Safety precautions included use of protective clothing, gloves, masks and eye protection (glasses). |
| Test Item Analysis | : Determination and documentation of the identity, strength, purity, stability and uniformity of the test item as defined in the Good Laboratory Practice (GLP) regulations, is the responsibility of the Sponsor. The Sponsor provided these test item characterization data (A Certificate of Analysis) for review by the Study Director and inclusion in the final report. |
| Stability Data | : Information on file with the Sponsor |
| Storage Condition | : Test Item and prepared solution(s)/formulation(s) were stored at ambient temperature. |
| Preparation of Test Item | : Test item was dissolved in distilled water. The formulation was prepared fresh on the day of dosing. The test item was administered in the dose volume of 10 ml/kg body weight. Actual preparation procedures are documented in the raw data. |
| Vehicle | : Distilled water |

Disclaimer :

The above physiochemical data of test substance is supplied by the Sponsor. All responsibility with regards to the accuracy and authenticity of this information remains with the Sponsor. The test lab is not responsible for any variations with the batch number supplied.

## Test System

**Species:** Rat

**Strain:** Sprague Dawley

**Sex**: Females

**Number**: Three females were used at each step.

**Source:** National Institute of Biosciences, Pune a registered source as approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

**Justification for selection of the rat as the test system:**

1) In order to meet the regulatory requirement for testing in a rodent species;
2) Widely used in as a species of choice for pre-clinical toxicological studies.
3) This strain is widely used throughout the industry in the non-clinical laboratory studies.
4) This study is intended to provide information on the health hazards likely to arise from short term accidental exposure to the test item by the oral route

**Selection of Animals**:

Healthy young adult females were used. Females were nulliparous and non- pregnant. The health examination was performed by a veterinarian. The animals of uniform body weight were selected. The individual body weight of the animals did not exceed ± 20% of group mean body weight. All animals were acclimatized to laboratory conditions for minimum of 5 days.

**Body Weight and Age**:

Female rats of the age of approximately 8 to 12 weeks old were used at the commencement of its dosing and its weight were within ± 20% of the mean weight of any animal used for dosing. Body weight range was 196.1 to 208.0 grams.

Body weights at the start :

Female Mean : 200.27 g (= 100 %)
Minimum : 196.1 g (- 2.08 %)
Maximum : 208.0 g (+ 3.86 %)
Total No. of animals : 6

**Identification**:

Each female rat was individually identified by the picric acid marking.

## Environmental Conditions

### Housing:

The rats were housed in polycarbonate cages.

### Room Air, Temperature and Humidity:

The animal room was independently provided with at least ten to fifteen air changes per hour of 100% fresh air that had been passed through the HEPA filters. Room temperature was maintained at 19.2 to 21.1 degree centigrade and room humidity was maintained at 55.2% to 58.2%.

### Light:

An artificial light and dark cycle of 12 hours each was provided to the room.

### Diet:

Rodent feed supplied by the Nutrivet Life Sciences, Pune, was provided *ad libitum* from individual feeders. Neither the Sponsor nor the Study Director was aware of any potential contaminant likely to be present in the diet that would interfere with the results of the study. The certificate of analysis provided by the supplier is appended to this report.

### Water:

Water was provided *ad libitum* from individual bottles attached to the cages. All water was from a local source and passed through the reverse osmosis membrane before use. Neither the Sponsor nor the study director was aware of any potential contaminant likely to be present in the water that would interfere with the results of the study.

## STUDY DESIGN

**Route:**

Oral (Gavage)

**Justification for Route of Administration:**

1) The dosage can be accurately administered;
2) Regulatory requirement.

**Duration of the study:**

A single dose was administered to each animal, followed by 14 days of observations.

**Experimental Design:**

| Dose Group | Dose (mg TOS/kg) | Concentration (mg/ml) | Dose volume (ml/kg) | Number of animals (Female) | |
|---|---|---|---|---|---|
| | | | | Step I | Step II |
| I | 2000 | 200 | 10 | 3 | - |
| I | 2000 | 200 | 10 | - | 3 |

**Justification for Selection of Doses:**

The doses were selected based on the available literature of the Test Item.

**Preparation of Animals:**

The animals were kept in their cages for at least 5 days prior to administration for acclimatization to the laboratory condition and after acclimatization period animals were randomly selected, marked with picric acid for individual identification.

**Administration of Test Item:**

The single dose of test item was administered to fasted rats (approximately 16 hours or more) by oral intubation, using a ball-tipped intubation needle fitted onto a syringe of appropriate size. Doses were calculated using recent (after fasting) body weights. 10 ml per kg of body weight was considered the maximum volume which could be administered to a rat.

Animals were given food 3-4 hours after test item administration.

## Type and Frequency of Tests, Analyses and Measurements

**Viability**:

Twice daily.

**Clinical Observations and General Appearance**:

Animals were observed for clinical signs, mortality until sacrifice.
Onset, duration and severity of any sign were recorded. The clinical signs and mortality observations were conducted at immediately (0 to 5 minutes), 5, 10, 30, 60 minutes, 2, 4 and 6 hours on the day of dosing and once daily thereafter for 14 day. Daily observation was done as far as possible at the same time.

**Body weights**:

Individual animal body weights were recorded, before fasting, after fasting prior to administration of the test item, weekly thereafter and at termination on day 14. Weight changes were calculated and recorded.

**Gross Pathology**:

Necropsy was performed on all animals at the end of the study period on day 15. Macroscopic examination of all the orifices, cavities and tissues were made and the findings were recorded. All animals surviving the study period were sacrificed by the carbon dioxide asphyxiation technique.

**Histopathology**:

No gross abnormalities were observed in animals sacrificed terminally hence, no histopathology was performed.

**Data and Reporting:**

Individual animal data is provided. In addition, tabulated mean values are provided for each parameter i.e. body weight, mortality, clinical signs of toxicity and gross pathology.

# RESULTS

## Clinical Signs of Toxicity and Mortality (Table No.I and Appendix No.I)

Group I

Step I :

Animals treated at the dose level of 2000 mg TOS/kg body weight did not result in any signs of toxicity during the study period of 14 days. All animals survived through the study period of 14 days.

Group I

Step II :

Animals treated at the dose level of 2000 mg TOS/kg body weight did not result in any signs of toxicity during the study period of 14 days. All animals survived through the study period of 14 days.

## Body Weight (Table No.II and Appendix No.II)

Group I

Step I (2000 mg TOS/kg) - Percent body weight gain after 7 days and 14 days was found to be 8.92% and 16.87% respectively.

Group I

Step II (2000 mg TOS/kg) - Percent body weight gain after 7 days and 14 days was found to be 8.46% and 16.49% respectively.

## Gross Pathological Findings (Table No.III and Appendix No.III)

Gross pathological examination did not reveal any abnormalities in animals from 2000 mg TOS/kg dose group.

## Regulatory References

1) OECD Principles of Good Laboratory Practice: Document # 1, ENV/MC/CHEM (98)17.

2) OECD Guideline for the Testing of Chemicals (No. 423, Section 4: Health Effects) "Acute Oral Toxicity -Acute Toxic Class Method " Adopted on 17th December 2001.

3) Recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines for Laboratory Animal Facility published in The Gazette of India, December 15, 1998.

## Table No. I

### Summary of Clinical Signs of Toxicity and Mortality

Laboratory Test Item Code : TAS/002/017

Test System : Sprague Dawley Rat

Sex : Female

Group I :

| Step No. | Dose mg TOS/kg | Observed Signs | Total Number of Animals | Animal Nos. | Period of signs in days From - to | Mortality |
|---|---|---|---|---|---|---|
| I | 2000 | Nil | 3 | 1 - 3 | 0 - 14 | 0/3 |

Group I :

| Step No. | Dose mg TOS/kg | Observed Signs | Total Number of Animals | Animal Nos. | Period of signs in days From - to | Mortality |
|---|---|---|---|---|---|---|
| II | 2000 | Nil | 3 | 4 - 6 | 0 - 14 | 0/3 |

**Table No.II**

**Mean Body Weight and Percent Body Weight Gain (g)**

Laboratory Test Item Code : TAS/002/017

Test System : Sprague Dawley Rat

Sex : Female

Group I :

| Step No. | Dose (mg TOS/kg body weight) | Before Fasting Body weight | Body weight Day 7 | % body weight gain day 0-7 | Body weight Day 14 | % body weight gain day 7- 14 | % body weight gain day 0- 14 |
|---|---|---|---|---|---|---|---|
| I | 2000 | 200.83 | 218.77 | 8.92 | 234.70 | 7.30 | 16.87 |

Group I :

| Step No. | Dose (mg TOS/kg body weight) | Before Fasting Body weight | Body weight Day 7 | % body weight gain day 0-7 | Body weight Day 14 | % body weight gain day 7- 14 | % body weight gain day 0- 14 |
|---|---|---|---|---|---|---|---|
| II | 2000 | 199.70 | 216.60 | 8.46 | 232.67 | 7.41 | 16.49 |

**Table No.III**

**Summary of Gross Pathological Findings**

Laboratory Test Item Code : TAS/002/017

Test System : Sprague Dawley Rat

Sex : Female

Group I :

| Step No. | Dose mg TOS/kg | Animal Numbers | Animal Fate | Gross Pathological Findings |
|---|---|---|---|---|
| I | 2000 | 1 - 3 | TS | No abnormality detected |

Group I :

| Step No. | Dose mg TOS/kg | Animal Numbers | Animal Fate | Gross Pathological Findings |
|---|---|---|---|---|
| II | 2000 | 4 - 6 | TS | No abnormality detected |

## Appendix No.I

### Individual Animal - Clinical Signs of Toxicity and Mortality

Laboratory Test Item Code : TAS/002/017

Test System : Sprague Dawley Rat

Sex : Female

Group I :

| Step No. | Dose mg TOS/ kg | Total Number of Animals | Observed Signs | Animal Nos. | Period of signs in days From - to | Mortality |
|---|---|---|---|---|---|---|
| I | 2000 | 3 | Nil | 1 | 0 - 14 | 0 |
| | | | | 2 | 0 - 14 | 0 |
| | | | | 3 | 0 - 14 | 0 |

Group I :

| Step No. | Dose mg TOS/ kg | Total Number of Animals | Observed Signs | Animal Nos. | Period of signs in days From - to | Mortality |
|---|---|---|---|---|---|---|
| II | 2000 | 3 | Nil | 4 | 0 - 14 | 0 |
| | | | | 5 | 0 - 14 | 0 |
| | | | | 6 | 0 - 14 | 0 |

## Appendix No.II

### Individual Animal - Body Weight and Percent Body Weight Gain (g)

Laboratory Test Item Code : TAS/002/017
Test System : Sprague Dawley Rat
Sex : Female

Group : I
Step I :
Dose : 2000 mg TOS/kg body weight

| Animal No. | Before Fasting Body weight | Body weight Day 7 | % body weight gain day 0-7 | Body weight Day 14 | % body weight gain day 7- 14 | % body weight gain day 0- 14 |
|---|---|---|---|---|---|---|
| 1 | 208.0 | 227.8 | 9.52 | 243.1 | 6.72 | 16.88 |
| 2 | 196.1 | 213.8 | 9.03 | 231.5 | 8.28 | 18.05 |
| 3 | 198.4 | 214.7 | 8.22 | 229.5 | 6.89 | 15.68 |

Group : I
Step II :
Dose : 2000 mg TOS/kg body weight

| Animal No. | Before Fasting Body weight | Body weight Day 7 | % body weight gain day 0-7 | Body weight Day 14 | % body weight gain day 7- 14 | % body weight gain day 0- 14 |
|---|---|---|---|---|---|---|
| 4 | 199.3 | 215.9 | 8.33 | 231.2 | 7.09 | 16.01 |
| 5 | 203.5 | 221.7 | 8.94 | 240.1 | 8.30 | 17.99 |
| 6 | 196.3 | 212.2 | 8.10 | 226.7 | 6.83 | 15.49 |

## Appendix No.III

### Individual Animal - Gross Pathological Findings

Laboratory Test Item Code : TAS/002/017
Test System : Sprague Dawley Rat
Sex : Female

Group : I
Step I :
Dose : 2000 mg TOS/kg body weight

| Animal No. | Fate | Gross Pathological Findings |
|---|---|---|
| 1 | TS | No abnormality detected |
| 2 | TS | No abnormality detected |
| 3 | TS | No abnormality detected |

Group : I
Step II :
Dose : 2000 mg TOS/kg body weight

| Animal No. | Fate | Gross Pathological Findings |
|---|---|---|
| 4 | TS | No abnormality detected |
| 5 | TS | No abnormality detected |
| 6 | TS | No abnormality detected |

TS = Terminal sacrifice

# ANNEXURE - I

## Certificate of Analysis - Test Item (1 Page) and Information on Correction Factor (1 Page)

**Advanced Enzyme Technologies Ltd.,**
Plot No. A-61/62, MIDC, Malegaon, Tal. Sinnar, Dist. Nashik - 422 113,
Maharashtra, India. Tel.: +91-99701 00750 / +91-2551-230 044, Fax: +91-2551-230 816
Email: info@enzymeindia.com, Web.: www.enzymeindia.com

## QUALITY ASSURANCE DEPARTMENT

## CERTIFICATE OF ANALYSIS

**PRODUCT NAME : CHITOSANASE**
**BATCH NO. : 061434**
**MFG. DATE : JUNE,2014**
**EXPIRY DATE : MAY,2016**

### PROTOCOL OF ANALYSIS

| TEST | RESULT | LIMITS |
|---|---|---|
| **Description** | Light brown coloured powder; having characteristics odour. | Light brown to brown cloured powder with characteristic odour. |
| **Solubility** | Soluble in Water : **Complies** | Soluble in Water. |
| **Lead** | **: Complies** | Not more than 5 ppm |
| **Microbial Limit-**<br>**Total viable count**<br>**Total coliforms/g**<br>**Escherichia.coli/25g**<br>**Salmonellae/25g** | <br>**: Complies**<br>**: Complies**<br>**: Complies**<br>**: Complies** | <br>NMT $1 \times 10^4$ cfu/g<br>Not more than 30<br>Negative by test<br>Negative by test |
| **Antimicrobial Activity** | Absent by test : **Complies** | Absent by test |
| **Chitosanase Activity** | 2,875 U/g | NLT 2,000 U/g |

**Remarks:** Sample **COMPLIES** as per Specifications.

**QA-CHEMIST**
**Date: October 28, 2014**

**MANAGE[illegible]Y ASSURANCE**

**Advanced Enzyme Technologies Ltd.,**
Plot No. A-61/62, MIDC, Malegaon, Tal. Sinnar, Dist. Nashik - 422 113,
Maharashtra, India. Tel.: +91-99701 00750 / +91-2551-230 044, Fax: +91-2551-230 816
Email: info@enzymeindia.com, Web.: www.enzymeindia.com

Date: 03/11/14
Place: Sinnar

**TO WHOMSOEVER IT MAY CONCERN**

Relevant information on the test material is presented below.

Sample: Spray-dried unformulated concentrate of Chitosanase

Source: Bacillus subtilis subsp. subtilis

| **Batch No.** | **061434** |
|---|---|
| Ash (%) | 7.76 |
| Water (%) | 7.25 |
| TOS (%)* | 84.99 |
| Activity (U/g) | 2875 |
| U/mg TOS | 3.38 |
| Protein (%) | 61.69 |
| Heavy Metals | Complies** |
| Mycotoxins | Complies** |

* Calculated
**As per JECFA specifications (http://www.fao.org/ag/agn/jecfa-additives/docs/enzymes_en.htm)

The above information is provided for conducting toxicology studies.

**MANAGER–QUALITY**
Advanced Enzyme Technologies Ltd
Sinnar, Nasik-422 113
Maharashtra, India

# ANNEXURE - II

## Feed Analysis (2 Pages)

# *Nutrivet Life Sciences*

*Nutrimix (Laboratory Animal Diets).*
*14, Ajay Apartment, Manikbaug, Sinhagad Road, Pune 411 051.* **Mob:- 09822006765,**
*mail – sdbhande@yahoo.com*

## CERTIFICATE OF ANALYSIS

**Name of the product: Rat / Mice Pelleted Diet.**
**Diet Code: Rat Std-1020 (Rodent Diet )**
**Description: A whitish brown coloured pellets**
**Date of Expiry : 4 months from date of Mfg**

**Date of Mfg. ; 20.09.2014**
**Date of Sampling : 21.09.2014**
**Date of Report : 24.09.2014**
**Batch No: 100009.**

### 1. Proximate analysis :

| No. | Test parameters | Results | Ranges |
|---|---|---|---|
| 1. | Moisture | 09.00 % | 10% Max. |
| 2. | Crude Protein | 20.10% | 17 – 22 % |
| 3. | Crude Fat | 03.20 % | 3 – 6 % |
| 4. | Crude fiber | 04.15 % | 3 - 7 % |
| 5. | Calcium | 00.96 % | 0.95 Mini. |
| 6. | Phosphorus | 00.67 % | 0.66 Mini. |
| 7. | Total ash | 06.12 % | 8.5% Max. |
| 8. | Carbohydrates | 57.27 % | 55 – 65 |
| 9. | Metabolizeble Energy ( kcal/gm ) | 02.90 | 2.8 - 3.2 |

### 2. Microbiological examination:

| No. | Test parameters | Result | Test method |
|---|---|---|---|
| 1. | Total Bacterial count (Cfu /gm ) | < 10 | AOAC 18th Cha -17. |
| 2. | Escherichia coli (Cfu /gm ) | < 10 | |
| 3. | Pseudomonas aeruginosa (Cfu /gm ) | < 10 | |
| 4. | Staphylococcus aureus (Cfu /gm ) | < 10 | |
| 6. | Total mould count (Cfu /gm ) | < 10 | |
| 7. | Aflatoxin (B1) | BDL | AOAC 975.36 |
| 8. | Aflatoxin (B2) | BDL | |

cfu - colony forming unit / BDL : Below Detectable Limits

Instructions : 1. Store the feed in cool, dry and well ventilated place off the floor.
2. Use within specified period.
3. Stop usage of feed if found defective.

(b) (4)

Quality Assurance
Mr. A. T. Rajgire

NUTRIVET LIFE SCIENCES Pune

(b) (4)

Technical Head / Lab In-charge
Dr.S. D.Bhande

# *Nutrivet Life Sciences*

*Nutrimix (Laboratory Animal Diets).*

*14, Ajay Apartment, Manikbaug, Sinhagad Road, Pune 411 051.* Mob:- 09822006765.

*mail – sdbhande@yahoo.com*


## CERTIFICATE OF ANALYSIS

**Name of the product: Rat / Mice Pelleted Diet.**
**Diet Code: Rat Std-1020 (Rodent Diet )**
**Description: A whitish brown coloured pellets**
**Date of Expiry : 4 months from date of Mfg**

**Date of Mfg. : 10.12.14**
**Date of Sampling : 11.12.14**
**Date of Report : 14.12.14**
**Batch No: 100012.**

**1. Proximate analysis :**

| No. | Test parameters | Results | Ranges |
|---|---|---|---|
| 1. | Moisture | 8.45 % | 10% Max. |
| 2. | Crude Protein | 20.00% | 17 - 22 % |
| 3. | Crude Fat | 3.50 % | 3 – 6 % |
| 4. | Crude fiber | 4.00 % | 3 - 7 % |
| 5. | Calcium | 0.96 % | 0.95 Mini. |
| 6. | Phosphorus | 0.67 % | 0.66 Mini |
| 7. | Total ash | 6.12 % | 8.5% Max. |
| 8. | Carbohydrates | 56.27 % | 55 – 65 |
| 9. | Metabolizeble Energy ( kcal/gm ) | 2.9 | 2.8 - 3.2 |

**2. Microbiological examination:**

| No. | Test parameters | Result | Test method |
|---|---|---|---|
| 1. | Total Bacterial count (Cfu /gm ) | < 10 | AOAC 18th Cha -17 |
| 2. | Escherichia coli (Cfu /gm ) | < 10 | |
| 3. | Pseudomonas aeruginosa (Cfu /gm ) | < 10 | |
| 4. | Staphylococcus aureus (Cfu /gm ) | < 10 | |
| 6. | Total mould count (Cfu /gm ) | < 10 | |
| 7. | Aflatoxin (B1) | BDL | AOAC 975.36 |
| 8. | Aflatoxin (B2) | BDL | |

cfu - colony forming unit / BDL : Below Detectable Limits

Instructions : 1. Store the feed in cool, dry and well ventilated place off the floor.
2. Use within specified period.
3. Stop usage of feed if found defective.

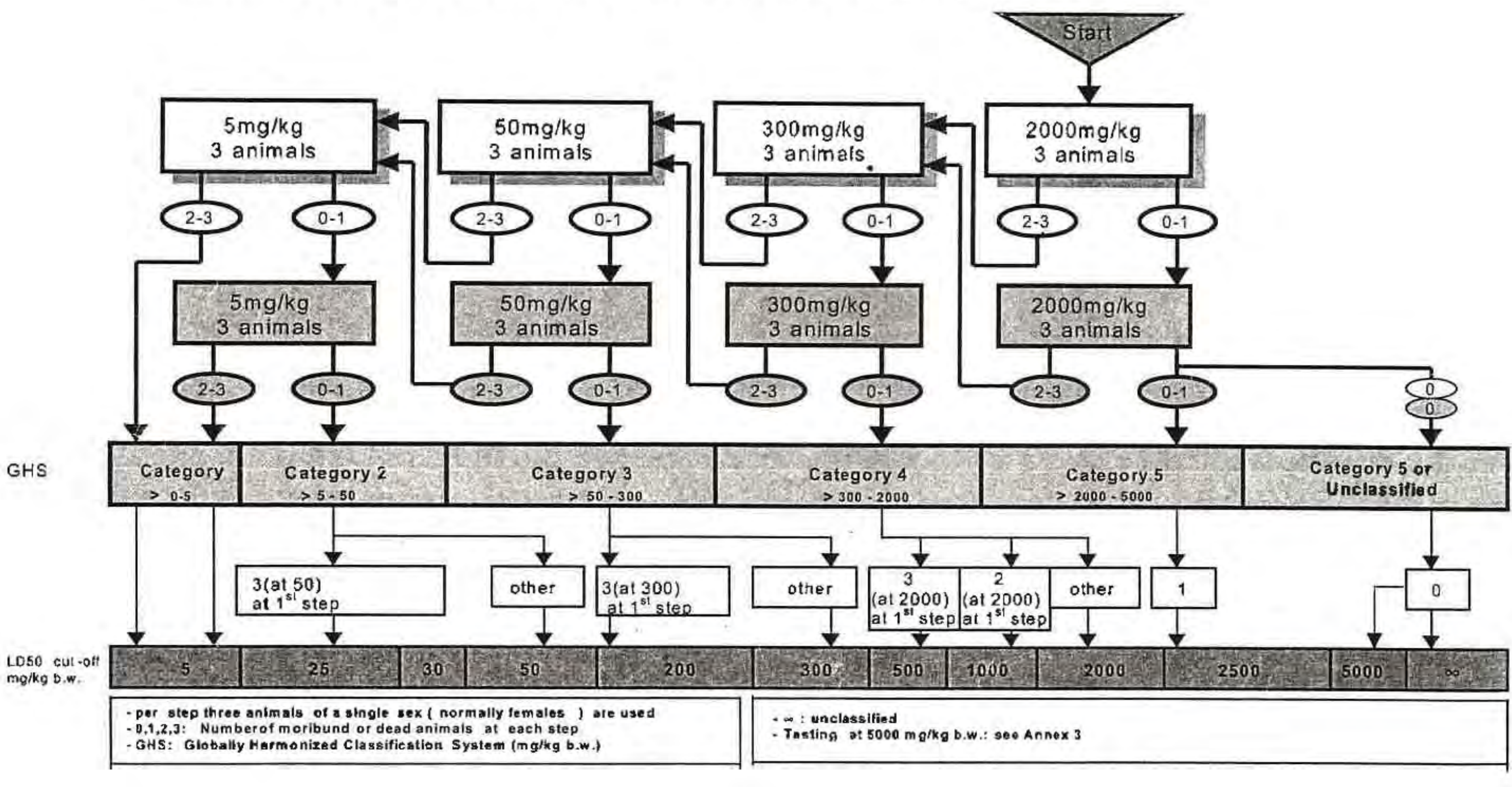
Quality Assurance
Mr. A. T. Rajgire

Technical Head / Lab In-charge
Dr. S. D. Bhande

# ANNEXURE - III

## Category Evaluation
(OECD Guideline 423,
Adopted on $17^{th}$ December 2001)
(1 Page)

ANNEX 2d: TEST PROCEDURE WITH A STARTING DOSE OF 2000 MG/KG BODY WEIGHT



# ANNEXURE - IV

## Certificate of Approval
## (Good Laboratory Practice, GLP)
## (1 Page)

# NATIONAL GLP COMPLIANCE MONITORING AUTHORITY

## *GLP CERTIFICATE*

GLP Inspection was carried out at **Indian Institute of Toxicology, 32 A/1, Hadapsar Industrial Estate, Pune – 411013, Maharashtra, INDIA** in the following areas of expertise:

- **Toxicity studies**
  - Acute Studies (Oral, Parenteral, Dermal, Inhalation, Dermal Irritation/ Corrosion, Eye Irritation/ Corrosion, Skin Sensitization Test)
  - Sub-acute Studies
  - Chronic Studies
- **Mutagenicity Studies**
  - Bacterial Reverse Mutation Assay (Ames Test)
  - Mammalian Erythrocyte Micronucleus Test
  - *In vivo/ In Vitro* Mammalian Chromosome Aberration Test

Based on the Inspection Report and the follow-up actions taken by the test facility, it is confirmed that the test facility is capable of conducting the above-mentioned tests/studies in compliance with OECD Principles of Good Laboratory Practice (GLP) for the types of chemicals and in test systems as listed below respectively:

Types of chemicals : Industrial Chemicals, Pesticides, Pharmaceuticals, Veterinary Drugs, Cosmetics, Food additives and Feed additives

Test Systems : Rat, Mice, Guinea pig, Rabbit, *Salmonella typhimurium*, tester strains viz. (TA 97a, TA 98, TA 100, TA 1535 & TA 102)

This GLP Certificate is valid for a period of three years from April 16, 2013, subject to the condition that the test facility complies with the Terms & Conditions of the National GLP Compliance Monitoring Authority's Document Number GLP-101.

**Certificate No. : GLP/C-046**

**Issue Date : 10-04-2013**

**(VINITA SHARMA)**
**Head**
**National GLP Compliance Monitoring Authority**
**Department of Science & Technology**
**Technology Bhavan New Delhi-110016**

# ANNEXURE - V

## Summary of Amendment(s) to the Study Plan (1 Page)

## Summary of Amendment(s) to the Study Plan

| Amendment Number | Amendment |
|---|---|
| 1 | The Study Schedule Dates finalized. |
| 2 | Experimental Completion Date added and Study Completion Date finalized. |

# INDIAN INSTITUTE OF TOXICOLOGY

भारतीय विषविज्ञान संस्था

FOUNDER DIRECTOR Late. DR. M. B. BHIDE (M.D.,F.C.A.I.)
Registered Office : "Kim" 2057, Sadashiv Peth, Pune - 411 030.
Correspondance Office : Flat No. 301, 401 "Usha-Shree" 90/124C, Erandawane, Gangote Street, Near Kamala Nehru Park, Pune - 411004.
Tel. Fax : (Testing Facility) : 020 - 2681 9961, 020 - 2681 9962 E-mail : iitoxicology@gmail.com

## FINAL REPORT

## (AMENDED ON 24-05-2016)

## IIT STUDY NUMBER 18103

## REPEATED DOSE 90-DAY ORAL TOXICITY STUDY WITH CHITOSANASE BY DAILY GAVAGE IN THE RAT FOLLOWED BY A 4 WEEK RECOVERY PERIOD

**STUDY DIRECTOR**
**Dr. R.M.Bhide Ph.D., ERT**

**TESTING FACILITY :**
**INDIAN INSTITUTE OF TOXICOLOGY**
**32 A/1, Hadapsar Industrial Estate,**
**Pune - 411 013.**

**SPONSOR'S REPRESENTATIVE**
**Mrs. Shilpa Risbud**

**SPONSOR ADDRESS :**
**ADVANCED ENZYME TECHNOLOGIES LTD.,**
**Sun Magnetica, 'A' wing, 5th Floor,**
**LIC Service Road,**
**Louiswadi, Thane (W) 400 604**
**Maharashtra, India.**

**REGULATORY REQUIREMENTS:**

OECD Guideline for the testing of Chemicals No. 408, "Repeated Dose-90 day Oral Toxicity Study in Rodents" adopted on 21st September, 1998.

### CONFIDENTIAL

This is a CONFIDENTIAL document. Any distribution beyond the parties listed within must be authorized by the sponsoring or requisitioning company/function. Reference to this document should only be made in documents having the same, or a higher, security classification.

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## STATEMENT OF GLP COMPLIANCE

| | |
|---|---|
| Title of the Study | : Repeated Dose 90-day Oral Toxicity Study with Chitosanase by daily gavage in the Rat followed by a 4 week recovery period. |
| Study Number | : 18103 |
| Study Plan Number | : SPL/002/060 |
| Name of the Test item | : Chitosanase |

The study was conducted in accordance with the Good Laboratory Practice Principles as Published by the OECD in 1998, No 1 ENV/MC/CHEM(98)17.

| Mr. V.M.Bhide M.B.A. | | 24.05.2016 |
|---|---|---|
| Test Facility Management | Signature | Date |

## STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE

| | |
|---|---|
| Title of the Study | : Repeated Dose 90-day Oral Toxicity Study with Chitosanase by daily gavage in the Rat followed by a 4 week recovery period. |
| Study Number | : 18103 |
| Study Plan Number | : SPL/002/060 |
| Name of the Test item | : Chitosanase |

The study was conducted in compliance to the Good Laboratory Principles as Published by OECD in 1998, No 1 ENV/MC/CHEM(98)17.

The study was conducted in compliance to the written Study Plan approved by the Study Director and authorized by the Sponsor and Indian Institute of Toxicology Management and all applicable Standard Operating Procedures of Indian Institute of Toxicology, Pune.

All original Raw Data including documentation, signed Study Plan, Study Plan Amendments, reserve sample, a copy of Final Report and specimen (wet tissue bottles, wax block, histopathology slides and smears for differential WBC count) are archived at Indian Institute of Toxicology, Pune.

I accept responsibility for the conduct and validity of the study and hereby declare that the study was conducted under my direction. This report is a complete, true and accurate record of the results obtained.

The report is amended with respect to the histopathological changes observed in lungs, spleen and kidneys and its relation to euthanasia process and were not of a biological significance (Page number 16 of 509) and the Study Completion date revised as 24-05-2016 after amendment was made.

The sponsor is responsible for Good laboratory Practice (GLP) compliance for all Test Item information and test item prepared formulation analyses and its report provided to the Indian Institute of Toxicology, Pune.

| Dr. R.M.Bhide Ph.D., ERT | | 24/5/2016 |
|---|---|---|
| Study Director | Signature | Date |

## STATEMENT OF QUALITY ASSURANCE UNIT

Title of the Study : Repeated Dose 90-day Oral Toxicity Study with Chitosanase by daily gavage in the Rat followed by a 4 week recovery period.

Study Number : 18103

Study Plan Number : SPL/002/060

Name of the Test item : Chitosanase

This study has been audited and the final report has been examined with respect to study plan, SOP and raw data. The report is true reflection of the raw data and the study was conducted in compliance with the principles of GLP. The audits were carried out according to the applicable SOP's of Quality Assurance Unit of Indian Institute of Toxicology, Pune. These reports are kept in the archives at Indian Institute of Toxicology, Pune.

Inspections were made by the Quality Assurance Unit of the Indian Institute of Toxicology for different phases of the study described in this report. The dates on which the inspections were made and the dates on which the findings were reported to the Study Director and to the facility Management are given below.

| Date(s) of Inspection | Phases Inspected | Date(s) findings reported to Study Director | Date(s) findings reported to Management |
|---|---|---|---|
| 16-12-2014 | Study Plan Review | 16-12-2014 | 16-12-2014 |
| 26-12-2014 | Pre-study Verification | 26-12-2014 | 26-12-2014 |
| 26-12-2014 | Amendment - 1 | 26-12-2014 | 26-12-2014 |
| 17-01-2015 | Pre-study Verification | 17-01-2015 | 17-01-2015 |
| 17-01-2015 | Amendment - 2 | 17-01-2015 | 17-01-2015 |
| 22-01-2015 | Randomization of Study Animals | 22-01-2015 | 22-01-2015 |
| 22-01-2015 | Ophthalmoscopic Examination | 22-01-2015 | 22-01-2015 |
| 23-01-2015 | Randomization of Study Animals | 23-01-2015 | 23-01-2015 |
| 23-01-2015 | Test item Preparation, Handling, Sampling, Labeling and Transport of Test Item for Analysis | 23-01-2015 | 23-01-2015 |
| 23-01-2015 | Test Item Administration - Gavage | 23-01-2015 | 23-01-2015 |
| 24-01-2015 | Test Item Administration - Gavage | 24-01-2015 | 24-01-2015 |
| 30-01-2015 | Body Weight | 30-01-2015 | 30-01-2015 |
| 06-02-2015 | Feed Consumption | 06-02-2015 | 06-02-2015 |
| 16-02-2015 | Clinical Signs Observation | 16-02-2015 | 16-02-2015 |
| 18-02-2015 | Test Item Administration - Gavage | 18-02-2015 | 18-02-2015 |
| 21-02-2015 | Safety / Environmental Monitoring | 21-02-2015 | 21-02-2015 |
| 07-03-2015 | Raw Data Audit (Interim) | 07-03-2015 | 07-03-2015 |
| 17-04-2015 | Test Item Preparation, Handling, Sampling, Labeling and Transport of Test Item for Analysis | 17-04-2015 | 17-04-2015 |
| 21-04-2015 | Urine Analysis | 21-04-2015 | 21-04-2015 |
| 23-04-2015 | Necropsy, Haematology | 23-04-2015 | 23-04-2015 |
| 24-04-2015 | Necropsy, Clinical Chemistry | 24-04-2015 | 24-04-2015 |
| 15-05-2015 | Histopathology Technique: Staining | 15-05-2015 | 15-05-2015 |
| 21-05-2015 | Necropsy | 21-05-2015 | 21-05-2015 |
| 22-05-2015 | Haematology, Clinical Chemistry | 22-05-2015 | 22-05-2015 |
| 06-06-2015 to 08-06-2015 | Raw Data Audit | 08-06-2015 | 08-06-2015 |
| 10-06-2015 to 12-06-2015 | Report Table Audit | 12-06-2015 | 12-06-2015 |
| 10-06-2015 to 12-06-2015 | Draft Report Audit | 12-06-2015 | 12-06-2015 |
| 29-06-2015 | Amendment - 3 | 29-06-2015 | 29-06-2015 |
| 30-06-2015 | Final Report Audit | 30-06-2015 | 30-06-2015 |
| 24-05-2016 | Final Report Amendment | 24-05-2016 | 24-05-2016 |
| 24-05-2016 | Amended Final Report Review | 24-05-2016 | 24-05-2016 |

Mrs. C.S.Bhide M.Sc. | | 24.5.2016

Quality Assurance Unit | Signature | Date

**LIST OF ABBREVIATIONS**

| | | |
|---|---|---|
| IIT | - | Indian Institute of Toxicology |
| GLP | - | Good Laboratory Practice |
| OECD | - | Organization for Economic Co-operation and Development |
| SOP | - | Standard Operating Procedure |
| G | - | Gram |
| mg | - | Milligram |
| mcg | - | Microgram |
| mg% | - | Milligram Percent |
| Kg | - | Kilogram |
| °C | - | Degree Celsius |
| % | - | Percent |
| No. | - | Number |
| Nos. | - | Numbers |
| ml | - | Milliliter |
| mm | - | Millimeter |
| e.g. | - | For Example |
| Ltd. | - | Limited |
| Contd. | - | Continued |
| TS | - | Terminal sacrifice |
| NAD | - | No abnormality detected |
| min. | - | Minutes |
| hrs. | - | Hours |
| Rev. | - | Reversal |
| EDTA | - | Ethylene Di-amine Tetra Acetic Acid |
| d/L | - | Deciliter |
| f/L | - | Fentoliter |
| µl | - | Micro liter |
| r.p.m. | - | Revolution Per Minute |
| $CO_2$ | - | Carbon Dioxide |
| i.e. | - | That is |
| IU/L | - | International Unit per Liter |
| U/L | - | Unit per Liter |
| mmol/l | - | Milimole per liter |
| pg | - | Pico gram |
| $\mu m^3$ | - | Cubic micro meter |
| Hb | - | Haemoglobin |
| RBC | - | Red Blood Corpuscles |
| HCT | - | Haematocrit |
| MCV | - | Mean Corpuscular Volume |
| MCH | - | Mean Corpuscular Haemoglobin |
| MCHC | - | Mean Corpuscular Haemoglobin Concentration |

## LIST OF ABBREVIATIONS (Contd.)

| | |
|---|---|
| WBC | - White Blood Corpuscles |
| N | - Neutrophils |
| L | - Lymphocytes |
| E | - Eosinophils |
| M | - Monocytes |
| B | - Basophils |
| Pt. | - Prothrombin time |
| ALT | - Alanine Aminotransferase |
| AST | - Aspartate Aminotransferase |
| ALP | - Alkaline Phosphatase |
| Sec. | - Second |
| TOS | - Total Solids |

# STUDY SCHEDULE

**Study Schedule:**

Study Initiation Date : 16-12-2014

**Dose Range Finding Study**

Experimental Starting Date : 06-01-2015

Day of First Dosing : 07-01-2015

Day of Last Dosing : 20-01-2015

Day of Necropsy ($15^{th}$ day) : 21-01-2015

Experimental Completion Date : 21-01-2015

**Main Study**

Experimental Starting Date : 22-01-2015

| | Male | Female |
|---|---|---|
| Day of First Dosing | : 23-01-2015 | 24-01-2015 |
| Day of Last Dosing | : 22-04-2015 | 23-04-2015 |
| Day of Necropsy ($91^{st}$ day) | : 23-04-2015 | 24-04-2015 |
| Day of Necropsy ($119^{th}$ day) | : 21-05-2015 | 22-05-2015 |

Experimental Completion Date : 06-06-2015

Study Completion Date : 24-05-2016

Date of Reporting : 24-05-2016

**PERSONNEL INVOLVED IN THE STUDY**

| | |
|---|---|
| Study Director | : Dr. R.M.Bhide Ph.D., ERT |
| Alternate Study Director | : Dr. R.P.Dighe Ph.D. |
| Veterinarian | : Dr. S.N.Khutale M.V.Sc. |
| Study Scientists | : Mr. M.P.Pawar B.Sc.<br>Miss S.V.Patil M.Sc.<br>Mr. D.D.Gawande M.Pharm.<br>Mr. M.P.Supekar M.Pharm.<br>Mr. J.M.Sonpetkar M.Pharm. |
| Pathology | : Dr. V.V.Dange M.V.Sc.<br>Dr. S.S.Kad M.V.Sc. |
| Clinical Pathology | : Miss S.K.Pise M.Sc. |
| Necropsy | : Dr. V.V.Dange M.V.Sc.<br>Dr. S.S.Kad M.V.Sc.<br>Mr. M.P.Pawar B.Sc.<br>Miss S.V.Patil M.Sc.<br>Dr. S.N.Khutale M.V.Sc.<br>Dr. P.U.Kore M.V.Sc.<br>Mr. D.D.Gawande M.Pharm. |
| Histology Techniques | : Dr. V.V.Dange M.V.Sc.<br>Miss S.K.Pise M.Sc.<br>Miss D.B.Survase M.Sc.<br>Dr. P.U.Kore M.V.Sc.<br>Mr. G.R.Palve |
| Statistics | : Mr. S.D.Nagpure B.Com.<br>Mr. D.K.Raut H.S.C. |
| Quality Assurance Unit | : Dr. P.R.Tikhe Ph.D.<br>Dr. R.M.Gosavi M.V.Sc.<br>Mrs. C.S.Bhide M.Sc. |
| Report Preparation | : Dr. R.M.Bhide Ph.D., ERT<br>Mr. S.D.Nagpure B.Com. |

## SUMMARY AND CONCLUSION

The Repeated Dose 90-day Oral Toxicity study followed by a 4 week recovery period was designed and conducted to determine the toxicity profile of **Chitosanase** when administered daily for 90 days in the Sprague Dawley rats.

**Chitosanase** was administered to animals at the dose levels of 250 mg TOS/kg, 500 mg TOS/kg and 1000 mg TOS/kg body weight. Two additional dose levels were added to the study as 0 mg TOS/kg (Rev.) and 1000 mg TOS/kg (Rev.), in order to study the reversibility or delayed occurrence of symptoms, if any. The control animals were administered with vehicle (distilled water) only. The doses were selected based on the results of the Acute Toxicity Study and Dose Range Finder Study conducted at Indian Institute of Toxicology, Pune.

Salient features of the study were as follows:

1) All the male and female animals from control and different dose groups up to 1000 mg TOS/kg survived throughout the dosing period of 90 days and the recovery period of 28 days.

2) Male and female animals from control and different dose groups exhibited normal body weight gain at the end of the dosing period of 90 days and the recovery period of 28 days.

3) Feed intake of animals from control and different dose groups was found to be comparable throughout the dosing period of 90 days and the recovery period of 28 days.

4) Ophthalmoscopic examination, conducted prior to and at the end of dosing period on animals from control and different dose groups did not reveal any abnormality.

5) No signs of toxicity were observed in male and female animals from different dose groups during the dosing period of 90 days and the recovery period of 28 days.

6) Detailed clinical observations conducted at weekly interval (upto $17^{th}$ week) did not reveal any abnormality in all male and female animals from control and different dose groups during the dosing period of 90 days and the recovery period of 28 days.

7) Towards the end of the exposure period in week 13, functional observation battery such as sensory reactivity to stimuli of different types (e.g. auditory, visual and proprioceptive stimuli) revealed no abnormalities attributable to the treatment.
Grip strength values observed in male and female animals for control and different dose groups were comparable.

Higher values of motor activity were observed in male animals from 500 mg TOS/kg dose group for first interval and lower values were observed in male animals from 1000 mg TOS/kg reversal dose group for third interval.
Higher values of motor activity were observed in female animals from 1000 mg TOS/kg dose group for first interval, higher values were observed in female animals from 500 mg TOS/kg and 1000 mg TOS/kg dose groups for third interval and lower values were observed in female animals from 1000 mg TOS/kg reversal dose group for first interval and higher values were observed from 1000 mg TOS/kg reversal dose group for third interval.
These changes were either not dose dependent or within normal laboratory range and were considered to be of no toxicological importance.
8) Haematological analysis in male and female animals conducted at the end of the dosing period on day 91, revealed statistically significant increase in the values of MCHC (1000 mg TOS/kg, male), MCV and MCH (250 mg TOS/kg, 500 mg TOS/kg and 1000 mg TOS/kg, male) and Prothrombin Time (1000 mg TOS/kg, female). In addition, statistically significant decrease was observed in the values of Hb and HCT (250 mg TOS/kg and 1000 mg TOS/kg, male), Total RBC and Platelets (250 mg TOS/kg, 500 mg TOS/kg and 1000 mg TOS/kg, male), MCH (250 mg TOS/kg, female) and Platelets (250 mg TOS/kg and 500 mg TOS/kg, female).
Haematological analysis in male animals conducted at the end of the recovery period on day 119, revealed statistically significant increase in the values of Hb, MCH and MCHC (1000 mg/kg, male).
Haematological analysis in female animals conducted at the end of the recovery period on day 119, revealed no statistically significant changes in the values of various parameters.

The increase/decrease in the values of various parameters was marginal and within the normal biological and laboratory limits.

9) Clinical biochemistry analysis in male and female animals conducted at the end of the dosing period on day 91, revealed statistically significant increase in the values of Chloride (500 mg TOS/kg and 1000 mg TOS/kg, male), Total Protein, Globulin, Potassium and Triglyceride (250 mg TOS/kg, female), Albumin (250 mg TOS/kg and 1000 mg TOS/kg, female), Sodium (250 mg TOS/kg, 500 mg TOS/kg and 1000 mg TOS/kg, female). In addition statistically significant decrease was observed in the values of Calcium (250 mg TOS/kg, 500 mg TOS/kg and 1000 mg TOS/kg, male), Sodium (500 mg TOS/kg and 1000 mg TOS/kg, male), Triglyceride (500 mg TOS/kg, male), Alkaline Phosphatase and Bilirubin (500 mg TOS/kg, female), Chloride (250 mg TOS/kg, 500 mg TOS/kg and 1000 mg TOS/kg, female),

At the end of the recovery period on day 119 (Reversal groups) statistically significant increase was observed in the values of Blood Urea Nitrogen and Urea Nitrogen (1000 mg TOS/kg, male), Chloride and triglyceride (1000 mg TOS/kg, female) and statistically significant decrease was observed in the values of Gamma Glutamyl Transferase (1000 mg TOS/kg, male).

The increase/decrease in the values of various parameters was marginal and within the normal biological and laboratory limits.

10) Urine analysis conducted during 13$^{th}$ and 17$^{th}$ week of dosing period (on day 86, 88, 89 and 119), revealed no abnormality attributable to the treatment.

11) At termination of dosing on day 91, organ weight data of male animals from different dose group, was found to be comparable with that of controls.

Organ weight data of male animals sacrificed on day 119 from 1000 mg TOS/kg reversal group, was found to be comparable with that of controls.

At termination of dosing on day 91, female animals from 500 mg TOS/kg dose group revealed increased relative weights of thymus. when compared with that of controls.

Organ weight data of female animals sacrificed on day 119 from 1000 mg TOS/kg reversal group, revealed decreased relative weights of liver and kidneys when compared with that of controls.

Although significant changes in the values of organ weight was observed in female animals from different dose groups, no related gross pathological and histopathological findings were seen, hence these findings were considered to be of no toxicological importance.

12) Gross pathological examination did not reveal any abnormality attributable to the treatment.

13) Histopathological examination did not reveal any abnormality attributable to the treatment. The findings observed in the control and high dose groups related to the haemorrhages in lungs, spleen and kidneys which were not a biological significance. These findings are related to the euthanasia process.

Based on these findings the No Observed Adverse Effect Level (NOAEL) of **Chitosanase** supplied by **Advanced Enzyme Technologies Ltd., Thane,** in the Sprague Dawley rat via oral route, over a period of 90 days was found to be 1000 mg TOS/kg body weight in male and female animals.

**DR. R.M.BHIDE** Ph.D., ERT
**STUDY DIRECTOR**

1) The results relate only to the items tested.
2) This report shall not be reproduced except in full, without the written approval of the laboratory.

# INDIAN INSTITUTE OF TOXICOLOGY

भारतीय विषविज्ञान संस्था

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## FINAL REPORT

## IIT STUDY NUMBER 18401

## BACTERIAL REVERSE MUTATION TEST OF CHITOSANASE

**STUDY DIRECTOR**
**DR. (MRS.) R.P.DIGHE Ph.D.**

**TESTING FACILITY :**
**INDIAN INSTITUTE OF TOXICOLOGY**
**32 A/1, Hadapsar Industrial Estate,**
**Pune - 411 013.**

**SPONSOR'S REPRESENTATIVE**
**Mrs. Shilpa Risbud**

**SPONSOR ADDRESS :**
**ADVANCED ENZYME TECHNOLOGIES LTD.,**
**Sun Magnetica, 'A' wing, 5th Floor,**
**LIC Service Road,**
**Louiswadi, Thane (W) 400 604**
**Maharashtra, India.**

**REGULATORY REQUIREMENTS:**

OECD Guideline for the Testing of Chemicals (No. 471, Section 4: Health Effects) "Bacterial Reverse Mutation Test" Adopted on 21st July 1997.

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## STATEMENT OF GLP COMPLIANCE

| | |
|---|---|
| Title of the Study | : Bacterial Reverse Mutation Test of Chitosanase |
| Study Number | : 18401 |
| Study Plan Number | : SPL/002/070 |
| Name of the Test item | : Chitosanase |

The study was conducted in accordance with the Good Laboratory Practice Principles as published by the OECD in 1998, No 1 ENV/MC/CHEM(98)17.

There were no circumstances that may have affected the quality or integrity of the study.

| Mr. V.M.Bhide M.B.A. | | 28.07.2015 |
|---|---|---|
| Test Facility Management | Signature | Date |

## STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE

| | |
|---|---|
| Title of the Study | : Bacterial Reverse Mutation Test of Chitosanase |
| Study Number | : 18401 |
| Study Plan Number | : SPL/002/070 |
| Name of the Test item | : Chitosanase |

The study was conducted in compliance to the Good Laboratory Practice Principles as Published by OECD in 1998, No 1 ENV/MC/CHEM(98)17.

The study was conducted in compliance to the written Study Plan approved by the Study Director and authorized by the Sponsor and Indian Institute of Toxicology Management and all applicable Standard Operating Procedures of Indian Institute of Toxicology, Pune.

All original Raw Data including documentation, signed Study Plan, Study Plan Amendments and a copy of final Report are archived at Indian Institute of Toxicology, Pune.

I accept responsibility for the conduct of the study and hereby declare that the study was conducted under my direction. This report is a complete true and accurate record of the results obtained.

The sponsor is responsible for Good laboratory Practice (GLP) compliance for all Test Item information unless determined by the Indian Institute of Toxicology.

| Dr. (Mrs.) R.P.Dighe Ph.D. | | 28/07/2015 |
|---|---|---|
| Study Director | Signature | Date |

## STATEMENT OF QUALITY ASSURANCE UNIT

Title of the Study : Bacterial Reverse Mutation Test of Chitosanase

Study Number : 18401

Study Plan Number : SPL/002/070

Name of the Test item : Chitosanase

This study has been audited and the final report has been examined with respect to study plan, SOP and raw data. The report is true reflection of the raw data and that the study was conducted in compliance with the principles of GLP. The audits were carried out according to the applicable SOP's of Quality Assurance Unit of Indian Institute of Toxicology, Pune. The report is kept in the archives at Indian Institute of Toxicology, Pune.

Inspections were made by the Quality Assurance Unit of the Indian Institute of Toxicology for different phases of the study described in this report. The dates on which the inspections were made and the dates on which the findings were reported to the Study Director and to the facility Management are given below. There were no minor/major findings that may have affected the quality or integrity of the study.

| Date(s) of Inspection | Phases Inspected | Date(s) findings reported to Study Director | Date(s) findings reported to Management |
|---|---|---|---|
| 30/06/2015 | Study Plan Review | 30/06/2015 | 30/06/2015 |
| 06/07/2015 | Pre Study Verification | 06/07/2015 | 06/07/2015 |
| 07/07/2015 | Dose Range Finding Study | 07/07/2015 | 07/07/2015 |
| 09/07/2015 | Performance of Bacterial Reverse Mutation Assay (Expt.I) | 09/07/2015 | 09/07/2015 |
| 11/07/2015 | Revertant Bacterial Colony Count (Expt.I) | 11/07/2015 | 11/07/2015 |
| 13/07/2015 | Revertant Bacterial Colony Count (Expt.II) | 13/07/2015 | 13/07/2015 |
| 14/07/2015 | Raw Data Audit | 14/07/2015 | 14/07/2015 |
| 20-07-2015 | Draft Report Audit | 20-07-2015 | 20-07-2015 |
| 27/07/2015 | Amendment 1 | 27/07/2015 | 27/07/2015 |
| 28/07/2015 | Final Report Audit | 28/07/2015 | 28/07/2015 |

Dr. P.R.Tikhe Ph.D. | | 28.07.2015
Quality Assurance Unit | Signature | Date

**PERSONNEL INVOLVED IN THE STUDY**

| | |
|---|---|
| Study Director | : Dr. R.P.Dighe Ph.D. |
| Study Scientist | : Mr. S.H.Oke M.S. |
| Statistics | : Mr. S.D.Nagpure B.Com.<br>Mr. D.K.Raut H.S.C. |
| Quality Assurance Unit | : Dr. P.R.Tikhe Ph.D.<br>Dr. R.M.Gosavi M.V.Sc. |
| Report Preparation | : Dr. R.P.Dighe Ph.D.<br>Mr. S.D.Nagpure B.Com. |

## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| % | - | Percent |
| °C | - | Degree Celsius |
| µg | - | Microgram |
| DRF | - | Dose Range Finding |
| Expt. | | Experiment |
| G-6-P | - | Glucose-6-Phosphate |
| GLP | - | Good Laboratory Practice |
| IIT | - | Indian Institute of Toxicology |
| KCL | - | Potassium Chloride |
| kg | - | Kilogram |
| M | - | Molar |
| mg | - | Milligram |
| $MgCl_2$ | - | Magnesium Chloride |
| ml | - | Milliliter |
| mm | - | Millimeter |
| NADP | - | Nicotiamide Adenine Dinucleotide Phosphate |
| No. | - | Number |
| Nos. | - | Numbers |
| OECD | - | Organization for Economic Co-operation and Development |
| PBS | - | Phosphate Buffer Saline |
| S9 | - | Supernatant at 9000 g |
| SD | - | Standard Deviation |
| SOP | - | Standard Operating Procedure |
| TOS | - | Total Solids |

## SUMMARY AND CONCLUSION

In order to determine the potential of the test item **Chitosanase** for its ability to induce gene mutations the Bacterial Reverse Mutation Test was conducted using *Salmonella typhimurium* tester strains viz. TA97a, TA 98, TA 100, TA 1535 and TA 102.

Initially, the Dose Range Finding study was conducted to evaluate the cytotoxicity of test item and/or its metabolites using TA 100 strain of *Salmonella typhimurium*. Cytotoxicity of test item was conducted in the range of 1 µg TOS/plate to 5000 µg TOS/plate concentrations of the test item with and without metabolic activation, using distilled water as vehicle. Parallel untreated controls and vehicle (negative) controls were tested simultaneously. No cytotoxicity was observed at and up to 5000 µg TOS/plate concentrations. Based on the results of the Dose Range Finding study, the concentrations of test item selected for Main test were 61.72 µg TOS/plate, 185.18 µg TOS/plate, 555.55 µg TOS/plate, 1666.66 µg TOS/plate and 5000 µg TOS/plate.

Distilled water was employed as the vehicle for preparation of different concentrations of the test item. No precipitation of the test item was observed at any test item concentrations in any of the tester strain(s) used in the study. The study was conducted without and with metabolic activation (S9 fraction) prepared from combination of sodium phenobarbital ando β-naphthoflavone induced rat liver. Untreated control, vehicle control (negative control) and appropriate positive controls (9-Aminoacridine, Sodium azide, 2-Nitrofluorene, Mitomycin C for without metabolic activation and Benzo(a)pyrene and 2-Aminoanthracene for with metabolic activation) were tested simultaneously. Two independent experiments were carried out using each tester strain with plating in triplicates at each concentration.

The results revealed that the mean numbers of revertant colonies counted at different concentrations of test item were comparable to that of the negative controls in both the experiments, in the absence and presence of metabolic activation. The number of revertant colonies in the positive controls increased by 1.90 to 52.79 fold under identical conditions.

**Conclusion**

Based on these results it is concluded that **Chitosanase** supplied by **Advanced Enzyme Technologies Ltd., Thane,** when tested at and up to 5000 µg TOS/plate concentration did not induce mutations in the presence and absence of microsomal enzymes (S-9 fraction) and is therefore non-mutagenic in this Bacterial Reverse Mutation Test.

**Dr. (Mrs.) R.P.Dighe**
**Study Director**

1) The results relate only to the items tested.
2) This report shall not be reproduced except in full, without the written approval of the laboratory.

## Schedule

Study Initiation Date : 30-06-2015

Experimental Starting Date : 07-07-2015

**Range Finding Study -**
Experimental Starting Date : 07-07-2015
Experiment Completion Date : 09-07-2015

**Definitive Study -**
**Experiment I**
Experimental Starting Date : 09-07-2015
Experiment Completion Date : 11-07-2015

**Experiment II**
Experimental Starting Date : 11-07-2015
Experiment Completion Date : 13-07-2015

Study Completion Date : 28-07-2015

Date of Reporting : 28-07-2015

## Archives

Raw data and study related documents generated during the course of the study at Indian Institute of Toxicology, together with a copy of the final report are lodged in the Indian Institute of Toxicology Archive.

Records will be retained for a period of nine years from the date of issue of the final report. At the end of the nine years retention period, the sponsor will be contacted and an advice may be sought on the future requirements.

## Test Item Return

On completion of the study and submission of the final report, all unused samples of the test item was returned to the Sponsor.

## Animal Welfare

All the recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines for Laboratory Animal Facility were followed for animal required for S9 preparation.

# OBJECTIVES

## Purpose

The bacterial reverse mutation test was commonly employed as an initial screen for genotoxic activity and in particular, for point mutation-inducing activity. This study was designed to evaluate **Chitosanase** and/or its metabolites for their ability to induce reverse mutation at the histidine locus in the genome of several strains of *Salmonella typhimurium* in the presence and absence of the rat microsomal enzymes (S-9).

# MATERIALS AND METHODS

## TEST ITEM

| | |
|---|---|
| Sponsor | : **Advanced Enzyme Technologies Ltd., Thane** |
| Laboratory Sponsor Code | : **SPN/002** |
| Test Item | : **Chitosanase** |
| Batch Number | : **061434** |
| Laboratory Test Item Code | : **TAS/002/017** |
| Manufacturing Date | : **June, 2014** |
| Expiry Date | : **May, 2016** |
| TOS (Total Solids) | : **84.99%** |
| Consistency | : **Solid powder** |
| Activity (Clinical Indication) | : **Biocatalyst for industry** |
| Safety Precautions | : Safety precautions included use of protective clothing, gloves, masks and eye protection (glasses). |
| Test Item Analysis | : Determination and documentation of the identity, strength, purity, stability and uniformity of the test item as defined in the Good Laboratory Practice (GLP) regulations, is the responsibility of the Sponsor. The Sponsor provided these test item characterization data (A Certificate of Analysis) for review by the Study Director and inclusion in the final report. |
| Stability Data | : Information on file. |
| Storage Condition | : Ambient temperature |

Dose Preparation :
The test item was administered with concentration(s) equivalent to 50 mg TOS/ml by applying the correction factor.
Test item was dissolved in distilled water to the required concentrations of the test item prior to exposure of cell cultures.
The volume of the vehicle was same in all cultures, including negative controls.
Test Item was freshly prepared on the day of experiment.
All records of the preparation procedures for test item was maintained in the raw data file.

Vehicle : Distilled water
Neither the Sponsor nor the Study Director was aware of any potential contaminants likely to be present in the vehicle that would interfere with the results of the study.

Disclaimer :

The above physiochemical data of test substance is supplied by the Sponsor. All responsibility with regards to the accuracy and authenticity of this information remains with the Sponsor. The test lab is not responsible for any variations with the batch number supplied.

## TEST SYSTEM

**Justification for Selection of the *Salmonella typhimurium* strain(s) as the Test System:**

1) In order to meet the regulatory requirement for genotoxicity testing,
2) Availability of comprehensive background data for all *salmonella* strains at the test facility,
3) Widely used as preferred *salmonella* strains of choice in genetic toxicological studies,
4) Widely used throughout the Industry as preferred strains of choice in genetic toxicological studies.

**Source:**

Strains were procured from Moltox Inc., USA and used in the study.

**Bacterial Strain:**

The following strains were used:
*S. typhimurium* TA 1535 hisG46 *rfa uvr*B
*S. typhimurium* TA 97a hisC3076 *rfa uvr*B pKM101
*S. typhimurium* TA 102 hisG428 pAQ1 *rfa* pKM101
*S. typhimurium* TA 98 hisD3052 *rfa uvr*B pKM101
*S. typhimurium* TA 100 hisG46 *rfa uvr*B pKM101

**Cell Density:** Number of cells per culture were 1-3 X $10^9$ cells/ml.

**Rationale for Selection of *Salmonella typhimurium* Strains:**

Salmonella Reverse Mutation Assay (Ames Test) detects reverse point mutations in the histidine operon, The histidine dependent bacteria becoming histidine (+) when a chemical induces mutations. Choice of the five tester strains has the advantage to detect both frame shift and base pair substitution mutations. TA 1535, TA 97a, TA 98 detect frame shift mutations TA 100, TA 102 base pair substitution mutations. The strain selection was that approved under the 1997 harmonization efforts of the OECD and the ICH and allows the use of the selected strains of bacteria.

**Storage**:

1) Frozen permanents stored at - 160°C in liquid nitrogen.
2) Master plates stored at 4° C.

## EXPERIMENTAL DESIGN

### Culture Conditions:

Following culture media were used in the assay:

1) Nutrient broth: Nutrient broth to grow overnight cultures.

2) Minimal glucose agar: Minimal Glucose Agar plates were incubated at 37°C for 24 hours after preparation to check the sterility of plates.

3) Top agar with histidine biotin solution: Top agar with trace of histidine and biotin for selection of histidine revertants.

Overnight Bacterial Cultures:

Each strain was inoculated in nutrient broth and incubated at 37°C. The cell count was measured. The cell count was 1 - 3 X $10^9$ cells/ml.

Fresh cultures of bacteria were grown up to the late exponential or early stationary phase of growth (approximately $10^9$ cells per ml). Cultures in late stationary phase were not used.

### Control Item:

Vehicle control (Negative control):

Distilled water was used with and without microsomal enzymes.

Positive Controls:

| Strain | S9 | Positive Mutagen |
|---|---|---|
| TA97a | - | 9-Aminoacridine |
| | + | Benzo(a)pyrene |
| TA98 | - | 2-Nitrofluorene |
| | + | 2-Aminoanthracene |
| TA100 | - | Sodium azide |
| | + | 2-Aminoanthracene |
| TA102 | - | Mitomycin C |
| | + | Benzo(a)pyrene |
| TA1535 | - | Sodium azide |
| | + | 2-Aminoanthracene |

Untreated Control (Spontaneous Revertant Data):

Bacterial cultures of tester strains were plated without and with metabolic activation only.

**Preparation of S-9 Mix:**

**Metabolic Activation:**

The liver homogenate S9 fraction was prepared on date 13-06-2015, batch number 14 was used in this study.

**Stimulation of Rat Liver Enzyme (Batch number 14):**

Mixed function oxidase system in the rat liver was stimulated following an intraperitoneal injection of sodium Phenobarbital of 80 mg/kg and oral administration of β-naphthoflavone at a dosage of 80 mg/kg/day for five consecutive days. On the 6th day, following an overnight fasting, the rats were killed and livers aseptically removed.

**Preparation of Liver Homogenate S-9:**

The preparation of enzyme was carried out with sterile glassware and solutions at 0 - 4 °C. Excised livers were transferred to beaker containing 0.15 M KCl (3 ml KCl : 1 g liver) minced with sterile scissors and homogenized. The homogenate was centrifuged for 10 minutes at 9000 x g and the supernatant divided into small aliquots. These were stored in liquid nitrogen and tested with mutagen Benzo(a)pyrine before use for the enzymatic activity of S-9 preparation.
The S-9 mix was prepared immediately prior to its use in the assay. The microsomal reaction mixture (S-9 mix) was contained the following component (D.M.Maron and B.N.Ames, (1983).

| For 10 ml | |
|---|---|
| S9 | 1.0 ml |
| $MgCl_2$-KCl Salt solution | 0.2 ml |
| 1 M G-6-P | 0.050 ml |
| 0.1 M NADP | 0.4 ml |
| 0.2 M Phosphate Buffer | 5.0 ml |
| Distilled water | 3.350 ml |

**Performance of Test:**

Plate incorporation assay:

The S-9 mix (0.5 ml) or phosphate buffered saline (PBS: pH 7.4), 0.1 ml of bacterial culture and 0.1 ml of the test item was added to sterile capped tubes containing molten top agar supplemented with histidine biotin solution.

This mixture was mixed and overlaid onto the surface of 25 ml of minimal bottom agar contained in 90 x 10 mm petri plate. After the top agar solidifies the plates were inverted and incubated for 48 or 72 hour at 37 °C. All plating was done in triplicate. The experiment was repeated once for consistent results.

**Solubility Test:**

Test item was dissolved in distilled water.

**Viability Test:**

Cell count of overnight culture was measured using heamocytometer by counting only viable cells. The required cell density was 1 - 3 X $10^9$ cells per ml.

**DOSE RANGE FINDING STUDY:**

1) Dose range finding study was performed using tester strain TA 100.

2) Tester strain TA 100 was used for cytotoxicity study because the growth inhibitory effect of tester strain TA 100 is generally representative of that observed on other tester strain. Similarly TA 100 tester strain shows comparatively high number of spontaneous revertants per plate, which also helps in gradation of cytotoxicity. Eight concentrations of test item ranging from 5000 to 1 µg TOS/plate were assessed.

**Main Test:**

**Criteria for Selection and Preparation of Concentrations for Main Test:**

No cytotoxicity was observed at and up to 5000 µg TOS/plate concentrations. Based on the results of the Dose Range Finding study, the concentrations of test item selected were 61.72 µg TOS/plate, 185.18 µg TOS/plate, 555.55 µg TOS/plate, 1666.66 µg TOS/plate and 5000 µg TOS/plate for the main test.

## Analyses and Measurements

### Scoring of Plates:

1) Bacterial background lawn.
Prior to counting the plates, condition of bacterial background lawn was observed for evidence of test item toxicity. The evidence of toxicity was compared with the vehicle control plate and recorded along with revertant counts for that plate.
2) Counting of revertant colonies.
Revertant colonies were counted manually.

### Data Presentation:

Data is presented in tabular form with respect to:

1) Number of revertants/plate

2) Mean number of revertants.

The standard deviation was calculated.

### Data Analysis:

The mean number of revertant colonies for all treatment groups were compared with vehicle control.

### Criteria for Mutagenic Potential of the Test Item:

The mutagenic activity of the test item was assessed by applying the following criteria.

1) If treatment with test item produces an increase in revertant colony numbers compared to concurrent vehicle controls with or without some evidence of positive dose relationship. Two fold increase in strains TA98, TA100 and TA102 and/or three fold increase in strains TA1535 and TA97a in two separate experiments, either in the presence or absence of S-9 mix in at least one strain will be considered to show evidence of mutagenic activity in this test system.

2) A test item will be considered non mutagenic if treatment with test item does not produce two fold (TA98, TA100 and TA102) and/or three fold (TA1535 and TA97a) increase in revertant colonies and does not show any dose response relationship in two separate experiments with any bacterial strain either with S-9 or without S-9 mix.

**Statistical Analysis, Evaluation and Interpretation of the Results:**

Mean number of Revertant colonies, ± SD and induced revertants were calculated.

The revertant colonies for untreated control, vehicle control and positive control groups fall within the in-house range of acceptable revertant colonies. Criteria to be considered for positive response was concentration related increase over the range tested and a reproducible increase at one or more concentrations in number of revertant colonies per plate in at least one strain with or without metabolic activation system. Biological relevance of results was considered.

Negative and equivocal results in the first trial were confirmed by a second trial, using the same method as specified above, with an alteration in concentration spacing and/or metabolic activation. Positive results were confirmed using the same experimental conditions.

The above-mentioned criteria were accepted for evaluation of results.

**Acceptance Criteria:**

A test was considered acceptable since the following occur:

- A confirmation of the bacterial genotype that was recently conducted.
- The tester strain cultures must exhibit a characteristic number of spontaneous revertants per plate. The number of spontaneous revertants was expected to fall within the spontaneous mutation rate of historical data at this facility. Although they may occasionally fall outside of this range, in such a case the experiment still was considered valid if the organisms respond normally to their respective positive controls.
- Acceptable positive control values demonstrated both the intrinsic sensitivity of the tester strains to mutagen exposure and the integrity of the S9-mix
- Historical vehicle and positive control values were used to assess the acceptability of the results.
- A minimum of four non-toxic test item dose levels were required to evaluate the assay data and none of these must show any signs of contamination.

## RESULTS

### Genotype Characterization

The tester strains fulfilled the quality check criteria.

## DOSE RANGE FINDING STUDY

### Background Lawn

The bacterial background lawn was comparable with that of the respective vehicle control plate up to the concentration of 5000 µg TOS/plate.

No cytotoxicity was observed up to 5000 µg TOS/plate concentration.

### Number of Revertants (Appendix No.I)

The mean number of histidine revertants in the vehicle control was 150.00 and 146.00 without and with metabolic activation, respectively. In the highest concentration of 5000 µg TOS/plate the mean number was 136.67, 136.67 without and with metabolic activation respectively in the TA 100 tester strain respectively which were comparable in comparison with respective vehicle controls. The mean numbers of revertants in the lower concentrations also were comparable with respective vehicle controls.

## MAIN TEST

### Background Lawn

The bacterial background lawn was comparable with that of the respective vehicle control plate up to the highest concentration of 5000 µg TOS/plate in all five tester strains.

**Number of Revertants**

**Experiment I (Table No.I and III)**

The mean number of histidine revertants in the vehicle control was TA 1535 : 9.00 (-S9), 11.00 (+S9); TA97a : 75.33 (-S9), 83.33 (+S9); TA 98 : 24.00 (-S9), 25.00 (+S9); TA 100 : 102.67 (-S9), 108.67 (+S9) and TA 102 : 248.67 (-S9), 264.00 (+S9). In the highest concentration of 5000 µg TOS/plate the mean number was TA 1535 : 12.67 (-S9), 13.67 (+S9); TA97a : 84.00 (-S9), 90.67 (+S9); TA 98 : 31.33 (-S9), 31.00 (+S9); TA 100 : 84.33 (-S9), 84.33 (+S9) and TA 102 : 304.00 (-S9), 314.00 (+S9) without (-S9) and with metabolic activation (+S9) respectively in the respective strain which were comparable in comparison with respective vehicle controls. The mean numbers of revertants in the lower concentrations also were comparable with respective vehicle controls, in TA1535, TA97a, TA98, TA100 and TA102 tester strains.

The parallel positive controls induced 4.12 to 51.63 (-S9) and 2.05 to 51.38 (+S9) fold revertants without and with metabolic activation in comparison with respective vehicle controls.

**Experiment II (Table No.II and IV)**

The mean number of histidine revertants in the vehicle control was TA 1535 : 9.00 (-S9), 11.00 (+S9); TA97a : 78.00 (-S9), 82.67 (+S9); TA 98 : 24.33 (-S9), 25.67 (+S9); TA 100 : 104.00 (-S9), 116.00 (+S9) and TA 102 : 272.67 (-S9), 283.33 (+S9). In the highest concentration of 5000 µg TOS/plate the mean number was TA 1535 : 11.00 (-S9), 13.00 (+S9); TA97a : 94.00 (-S9), 98.67 (+S9); TA 98 : 30.33 (-S9), 33.00 (+S9); TA 100 : 114.00 (-S9), 125.33 (+S9) and TA 102 : 311.33 (-S9), 334.00 (+S9) without (-S9) and with metabolic activation (+S9) respectively in the respective strain which were comparable in comparison with respective vehicle controls. The mean numbers of revertants in the lower concentrations also were comparable with respective vehicle controls, in TA1535, TA97a, TA98, TA100 and TA102 tester strains.

The parallel positive controls induced 2.99 to 49.40 (-S9) and 1.90 to 52.79 (+S9) fold revertants without and with metabolic activation in comparison with respective vehicle controls.

## REGULATORY REFERENCES

1) OECD Principles of Good Laboratory Practice: Document # 1, ENV/MC/CHEM (98)17.

2) OECD Guideline for the Testing of Chemicals (No. 471, Section 4: Health Effects) "Bacterial Reverse Mutation Test" Adopted on 21st July 1997.

3) Revised methods for the Salmonella mutagenicity test, D.M.Maron and B.N.Ames, Mutation Research, 113 pg 207 (1983).

4) Recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines for Laboratory Animal Facility published in The Gazette of India, December 15, 1998.

5) Mutagenicity Testing a practical approach : S.Venitt and J.M. Parry, IRL Press pg 45-97 (1984)

6) Guidance Document on Toxicology for Registration of Pesticides in India "Kanungo Committee Report", "Protocols on Toxicological Studies" and "Data Requirement" Insecticide Act 1968. Ministry of Agriculture Department of Agriculture & Co-operation Central Insecticides Board & Registration Committee Directorate of Plant Protection Quarantine & Storage NH-IV, Faridabad. Genotoxicity Studies - Bacterial Reverse Mutation Test (Ames Test) - 6 (i).

## TABLE NO.I

## COLONY COUNTS OF HISTIDINE REVERTANTS IN *SALMONELLA*

Laboratory Test Item Code : TAS/002/017
Test System : *Salmonella typhimurium*

**Strain : TA 1535** **Experiment No.: 1**

| Treatment | Group Number | Dose µg TOS/plate | Number of Revertant Colonies | | | | | | Induced Revertants |
|---|---|---|---|---|---|---|---|---|---|
| | | | S-9 | Individual Colony Counts | | | Mean | ± S.D. | |
| Test Item | I | 5000 | - | 13 | 11 | 14 | 12.67 | 1.53 | 3.67 |
| | | | + | 12 | 15 | 14 | 13.67 | 1.53 | 2.67 |
| | II | 1666.66 | - | 11 | 12 | 13 | 12.00 | 1.00 | 3.00 |
| | | | + | 15 | 14 | 13 | 14.00 | 1.00 | 3.00 |
| | III | 555.55 | - | 9 | 10 | 13 | 10.67 | 2.08 | 1.67 |
| | | | + | 14 | 9 | 11 | 11.33 | 2.52 | 0.33 |
| | IV | 185.18 | - | 11 | 11 | 11 | 11.00 | 0.00 | 2.00 |
| | | | + | 14 | 12 | 13 | 13.00 | 1.00 | 2.00 |
| | V | 61.72 | - | 9 | 11 | 12 | 10.67 | 1.53 | 1.67 |
| | | | + | 10 | 11 | 13 | 11.33 | 1.53 | 0.33 |
| Distilled Water (Solvent Control) | VI | 100 µl | - | 9 | 8 | 10 | 9.00 | 1.00 | 0.00 |
| | | | + | 11 | 12 | 10 | 11.00 | 1.00 | 0.00 |
| Sodium Azide | VII | 2.0 | - | 426 | 512 | 456 | 464.67 | 43.65 | 455.67 |
| 2-Aminoanthracene (Positive Control) | | 0.5 | + | 374 | 312 | 338 | 341.33 | 31.13 | 330.33 |
| Untreated Control | VIII | - | - | 8 | 8 | 10 | 8.67 | 1.15 | -0.33 |
| | | | + | 9 | 9 | 10 | 9.33 | 0.58 | -1.67 |

## TABLE NO.I (Contd.)

## COLONY COUNTS OF HISTIDINE REVERTANTS IN *SALMONELLA*

Laboratory Test Item Code : TAS/002/017
Test System : *Salmonella typhimurium*

**Strain : TA 97a** **Experiment No.: 1**

| Treatment | Group Number | Dose µg TOS/plate | Number of Revertant Colonies | | | | | | Induced Revertants |
|---|---|---|---|---|---|---|---|---|---|
| | | | S-9 | Individual Colony Counts | | | Mean | ± S.D. | |
| Test Item | I | 5000 | - | 87 | 81 | 84 | 84.00 | 3.00 | 8.67 |
| | | | + | 88 | 91 | 93 | 90.67 | 2.52 | 7.33 |
| | II | 1666.66 | - | 74 | 76 | 79 | 76.33 | 2.52 | 1.00 |
| | | | + | 83 | 82 | 84 | 83.00 | 1.00 | -0.33 |
| | III | 555.55 | - | 91 | 97 | 81 | 89.67 | 8.08 | 14.33 |
| | | | + | 94 | 81 | 87 | 87.33 | 6.51 | 4.00 |
| | IV | 185.18 | - | 84 | 82 | 86 | 84.00 | 2.00 | 8.67 |
| | | | + | 86 | 89 | 83 | 86.00 | 3.00 | 2.67 |
| | V | 61.72 | - | 88 | 89 | 91 | 89.33 | 1.53 | 14.00 |
| | | | + | 93 | 91 | 86 | 90.00 | 3.61 | 6.67 |
| Distilled Water (Solvent Control) | VI | 100 µl | - | 71 | 74 | 81 | 75.33 | 5.13 | 0.00 |
| | | | + | 84 | 87 | 79 | 83.33 | 4.04 | 0.00 |
| 9-Aminoacridine | VII | 50 | - | 298 | 346 | 288 | 310.67 | 31.01 | 235.33 |
| Benzo(a)pyrene (Positive Control) | | 2.5 | + | 608 | 576 | 502 | 562.00 | 54.37 | 478.67 |
| Untreated Control | VIII | - | - | 73 | 72 | 77 | 74.00 | 2.65 | -1.33 |
| | | | + | 79 | 77 | 80 | 78.67 | 1.53 | -4.67 |

## TABLE NO.1 (Contd.)

## COLONY COUNTS OF HISTIDINE REVERTANTS IN *SALMONELLA*

Laboratory Test Item Code : TAS/002/017
Test System : *Salmonella typhimurium*

**Strain : TA 98** **Experiment No.: 1**

| Treatment | Group Number | Dose µg TOS/plate | Number of Revertant Colonies | | | | | | Induced Revertants |
|---|---|---|---|---|---|---|---|---|---|
| | | | S-9 | Individual Colony Counts | | | Mean | ± S.D. | |
| Test Item | I | 5000 | - | 28 | 32 | 34 | 31.33 | 3.06 | 7.33 |
| | | | + | 27 | 33 | 33 | 31.00 | 3.46 | 6.00 |
| | II | 1666.66 | - | 24 | 27 | 29 | 26.67 | 2.52 | 2.67 |
| | | | + | 28 | 27 | 32 | 29.00 | 2.65 | 4.00 |
| | III | 555.55 | - | 26 | 28 | 29 | 27.67 | 1.53 | 3.67 |
| | | | + | 29 | 32 | 34 | 31.67 | 2.52 | 6.67 |
| | IV | 185.18 | - | 33 | 30 | 25 | 29.33 | 4.04 | 5.33 |
| | | | + | 28 | 33 | 31 | 30.67 | 2.52 | 5.67 |
| | V | 61.72 | - | 27 | 24 | 29 | 26.67 | 2.52 | 2.67 |
| | | | + | 29 | 26 | 27 | 27.33 | 1.53 | 2.33 |
| Distilled Water (Solvent Control) | VI | 100 µl | - | 23 | 25 | 24 | 24.00 | 1.00 | 0.00 |
| | | | + | 24 | 27 | 24 | 25.00 | 1.73 | 0.00 |
| 2-Nitrofluorene | VII | 25 | - | 872 | 1028 | 988 | 962.67 | 81.03 | 938.67 |
| 2-Aminoanthracene (Positive Control) | | 5 | + | 1172 | 1384 | 1298 | 1284.67 | 106.63 | 1259.67 |
| Untreated Control | VIII | - | - | 20 | 19 | 22 | 20.33 | 1.53 | -3.67 |
| | | | + | 22 | 20 | 22 | 21.33 | 1.15 | -3.67 |

## TABLE NO.I (Contd.)

## COLONY COUNTS OF HISTIDINE REVERTANTS IN *SALMONELLA*

Laboratory Test Item Code : TAS/002/017
Test System : *Salmonella typhimurium*

**Strain : TA 100** **Experiment No.: 1**

| Treatment | Group Number | Dose µg TOS/plate | Number of Revertant Colonies | | | | | | Induced Revertants |
|---|---|---|---|---|---|---|---|---|---|
| | | | S-9 | Individual Colony Counts | | | Mean | ± S.D. | |
| Test Item | I | 5000 | - | 91 | 79 | 83 | 84.33 | 6.11 | -18.33 |
| | | | + | 87 | 84 | 82 | 84.33 | 2.52 | -24.33 |
| | II | 1666.66 | - | 120 | 144 | 100 | 121.33 | 22.03 | 18.67 |
| | | | + | 117 | 124 | 118 | 119.67 | 3.79 | 11.00 |
| | III | 555.55 | - | 118 | 112 | 113 | 114.33 | 3.21 | 11.67 |
| | | | + | 117 | 114 | 116 | 115.67 | 1.53 | 7.00 |
| | IV | 185.18 | - | 128 | 114 | 127 | 123.00 | 7.81 | 20.33 |
| | | | + | 130 | 118 | 130 | 126.00 | 6.93 | 17.33 |
| | V | 61.72 | - | 132 | 134 | 136 | 134.00 | 2.00 | 31.33 |
| | | | + | 128 | 128 | 138 | 131.33 | 5.77 | 22.67 |
| Distilled Water (Solvent Control) | VI | 100 µl | - | 102 | 108 | 98 | 102.67 | 5.03 | 0.00 |
| | | | + | 114 | 112 | 100 | 108.67 | 7.57 | 0.00 |
| Sodium azide | VII | 0.5 | - | 924 | 898 | 890 | 904.00 | 17.78 | 801.33 |
| 2-Aminoanthracene (Positive Control) | | 1 | + | 988 | 952 | 888 | 942.67 | 50.65 | 834.00 |
| Untreated Control | VIII | - | - | 74 | 76 | 78 | 76.00 | 2.00 | -26.67 |
| | | | + | 84 | 80 | 79 | 81.00 | 2.65 | -27.67 |

## TABLE NO.I (Contd.)

## COLONY COUNTS OF HISTIDINE REVERTANTS IN *SALMONELLA*

Laboratory Test Item Code : TAS/002/017
Test System : *Salmonella typhimurium*

**Strain : TA 102** **Experiment No.: 1**

| Treatment | Group Number | Dose µg TOS/plate | Number of Revertant Colonies | | | | | | Induced Revertants |
|---|---|---|---|---|---|---|---|---|---|
| | | | S-9 | Individual Colony Counts | | | Mean | ± S.D. | |
| Test Item | I | 5000 | - | 280 | 320 | 312 | 304.00 | 21.17 | 55.33 |
| | | | + | 272 | 328 | 342 | 314.00 | 37.04 | 50.00 |
| | II | 1666.66 | - | 274 | 296 | 304 | 291.33 | 15.53 | 42.67 |
| | | | + | 288 | 306 | 298 | 297.33 | 9.02 | 33.33 |
| | III | 555.55 | - | 304 | 326 | 328 | 319.33 | 13.32 | 70.67 |
| | | | + | 324 | 318 | 290 | 310.67 | 18.15 | 46.67 |
| | IV | 185.18 | - | 294 | 280 | 288 | 287.33 | 7.02 | 38.67 |
| | | | + | 296 | 288 | 296 | 293.33 | 4.62 | 29.33 |
| | V | 61.72 | - | 280 | 276 | 280 | 278.67 | 2.31 | 30.00 |
| | | | + | 282 | 278 | 298 | 286.00 | 10.58 | 22.00 |
| Distilled Water (Solvent Control) | VI | 100 µl | - | 240 | 248 | 258 | 248.67 | 9.02 | 0.00 |
| | | | + | 270 | 264 | 258 | 264.00 | 6.00 | 0.00 |
| Mitomycin C | VII | 0.25 | - | 838 | 942 | 894 | 891.33 | 52.05 | 642.67 |
| Benzo(a)pyrene (Positive Control) | | 2.5 | + | 512 | 518 | 596 | 542.00 | 46.86 | 278.00 |
| Untreated Control | VIII | - | - | 220 | 240 | 228 | 229.33 | 10.07 | -19.33 |
| | | | + | 240 | 322 | 236 | 266.00 | 48.54 | 2.00 |

## TABLE NO.II

## COLONY COUNTS OF HISTIDINE REVERTANTS IN *SALMONELLA*

Laboratory Test Item Code : TAS/002/017
Test System : *Salmonella typhimurium*

**Strain : TA 1535** **Experiment No.: 2**

| Treatment | Group Number | Dose µg TOS/plate | Number of Revertant Colonies | | | | | | Induced Revertants |
|---|---|---|---|---|---|---|---|---|---|
| | | | S-9 | Individual Colony Counts | | | Mean | ± S.D. | |
| Test Item | I | 5000 | - | 10 | 11 | 12 | 11.00 | 1.00 | 2.00 |
| | | | + | 12 | 14 | 13 | 13.00 | 1.00 | 2.00 |
| | II | 1666.66 | - | 11 | 11 | 14 | 12.00 | 1.73 | 3.00 |
| | | | + | 14 | 13 | 12 | 13.00 | 1.00 | 2.00 |
| | III | 555.55 | - | 9 | 9 | 12 | 10.00 | 1.73 | 1.00 |
| | | | + | 14 | 12 | 11 | 12.33 | 1.53 | 1.33 |
| | IV | 185.18 | - | 11 | 11 | 12 | 11.33 | 0.58 | 2.33 |
| | | | + | 14 | 12 | 13 | 13.00 | 1.00 | 2.00 |
| | V | 61.72 | - | 9 | 11 | 12 | 10.67 | 1.53 | 1.67 |
| | | | + | 10 | 11 | 13 | 11.33 | 1.53 | 0.33 |
| Distilled Water (Solvent Control) | VI | 100 µl | - | 9 | 8 | 10 | 9.00 | 1.00 | 0.00 |
| | | | + | 11 | 12 | 10 | 11.00 | 1.00 | 0.00 |
| Sodium Azide | VII | 2 | - | 420 | 434 | 480 | 444.67 | 31.39 | 435.67 |
| 2-Aminoanthracene (Positive Control) | | 0.5 | + | 358 | 364 | 372 | 364.67 | 7.02 | 353.67 |
| Untreated Control | VIII | - | - | 9 | 8 | 8 | 8.33 | 0.58 | -0.67 |
| | | | + | 10 | 9 | 8 | 9.00 | 1.00 | -2.00 |

## TABLE NO.II (Contd.)

## COLONY COUNTS OF HISTIDINE REVERTANTS IN *SALMONELLA*

Laboratory Test Item Code : TAS/002/017
Test System : *Salmonella typhimurium*

**Strain : TA 97a** **Experiment No.: 2**

| Treatment | Group Number | Dose µg TOS/plate | Number of Revertant Colonies | | | | | | Induced Revertants |
|---|---|---|---|---|---|---|---|---|---|
| | | | S-9 | Individual Colony Counts | | | Mean | ± S.D. | |
| Test Item | I | 5000 | - | 97 | 94 | 91 | 94.00 | 3.00 | 16.00 |
| | | | + | 98 | 102 | 96 | 98.67 | 3.06 | 16.00 |
| | II | 1666.66 | - | 95 | 87 | 84 | 88.67 | 5.69 | 10.67 |
| | | | + | 97 | 94 | 93 | 94.67 | 2.08 | 12.00 |
| | III | 555.55 | - | 88 | 89 | 87 | 88.00 | 1.00 | 10.00 |
| | | | + | 89 | 91 | 93 | 91.00 | 2.00 | 8.33 |
| | IV | 185.18 | - | 104 | 98 | 94 | 98.67 | 5.03 | 20.67 |
| | | | + | 98 | 96 | 97 | 97.00 | 1.00 | 14.33 |
| | V | 61.72 | - | 94 | 81 | 88 | 87.67 | 6.51 | 9.67 |
| | | | + | 89 | 91 | 89 | 89.67 | 1.15 | 7.00 |
| Distilled Water (Solvent Control) | VI | 100 µl | - | 78 | 84 | 72 | 78.00 | 6.00 | 0.00 |
| | | | + | 74 | 82 | 92 | 82.67 | 9.02 | 0.00 |
| 9-Aminoacridine | VII | 50 | - | 312 | 328 | 290 | 310.00 | 19.08 | 232.00 |
| Benzo(a)pyrene (Positive Control) | | 2.5 | + | 584 | 498 | 536 | 539.33 | 43.10 | 456.67 |
| Untreated Control | VIII | - | - | 72 | 75 | 74 | 73.67 | 1.53 | -4.33 |
| | | | + | 74 | 76 | 72 | 74.00 | 2.00 | -8.67 |

## TABLE NO.II (Contd.)

## COLONY COUNTS OF HISTIDINE REVERTANTS IN *SALMONELLA*

Laboratory Test Item Code : TAS/002/017
Test System : *Salmonella typhimurium*

**Strain : TA 98** **Experiment No.: 2**

| Treatment | Group Number | Dose µg TOS/plate | Number of Revertant Colonies | | | | | | Induced Revertants |
|---|---|---|---|---|---|---|---|---|---|
| | | | S-9 | Individual Colony Counts | | | Mean | ± S.D. | |
| Test Item | I | 5000 | - | 31 | 32 | 28 | 30.33 | 2.08 | 6.00 |
| | | | + | 33 | 34 | 32 | 33.00 | 1.00 | 7.33 |
| | II | 1666.66 | - | 34 | 35 | 29 | 32.67 | 3.21 | 8.33 |
| | | | + | 28 | 35 | 35 | 32.67 | 4.04 | 7.00 |
| | III | 555.55 | - | 27 | 26 | 28 | 27.00 | 1.00 | 2.67 |
| | | | + | 30 | 29 | 28 | 29.00 | 1.00 | 3.33 |
| | IV | 185.18 | - | 27 | 24 | 28 | 26.33 | 2.08 | 2.00 |
| | | | + | 28 | 28 | 29 | 28.33 | 0.58 | 2.67 |
| | V | 61.72 | - | 30 | 29 | 24 | 27.67 | 3.21 | 3.33 |
| | | | + | 28 | 27 | 32 | 29.00 | 2.65 | 3.33 |
| Distilled Water (Solvent Control) | VI | 100 µl | - | 24 | 26 | 23 | 24.33 | 1.53 | 0.00 |
| | | | + | 22 | 28 | 27 | 25.67 | 3.21 | 0.00 |
| 2-Nitrofluorene | VII | 25 | - | 912 | 926 | 872 | 903.33 | 28.02 | 879.00 |
| 2-Aminoanthracene (Positive Control) | | 5 | + | 1284 | 1372 | 1410 | 1355.33 | 64.63 | 1329.67 |
| Untreated Control | VIII | - | - | 20 | 22 | 22 | 21.33 | 1.15 | -3.00 |
| | | | + | 23 | 24 | 23 | 23.33 | 0.58 | -2.33 |

## TABLE NO.II (Contd.)

## COLONY COUNTS OF HISTIDINE REVERTANTS IN *SALMONELLA*

Laboratory Test Item Code : TAS/002/017
Test System : *Salmonella typhimurium*

**Strain : TA 100** **Experiment No.: 2**

| Treatment | Group Number | Dose µg TOS/plate | Number of Revertant Colonies | | | | | | Induced Revertants |
|---|---|---|---|---|---|---|---|---|---|
| | | | S-9 | Individual Colony Counts | | | Mean | ± S.D. | |
| Test Item | I | 5000 | - | 104 | 116 | 122 | 114.00 | 9.17 | 10.00 |
| | | | + | 127 | 124 | 125 | 125.33 | 1.53 | 9.33 |
| | II | 1666.66 | - | 130 | 134 | 136 | 133.33 | 3.06 | 29.33 |
| | | | + | 140 | 142 | 132 | 138.00 | 5.29 | 22.00 |
| | III | 555.55 | - | 120 | 124 | 128 | 124.00 | 4.00 | 20.00 |
| | | | + | 122 | 126 | 130 | 126.00 | 4.00 | 10.00 |
| | IV | 185.18 | - | 120 | 118 | 106 | 114.67 | 7.57 | 10.67 |
| | | | + | 124 | 120 | 112 | 118.67 | 6.11 | 2.67 |
| | V | 61.72 | - | 98 | 114 | 118 | 110.00 | 10.58 | 6.00 |
| | | | + | 98 | 120 | 116 | 111.33 | 11.72 | -4.67 |
| Distilled Water (Solvent Control) | VI | 100 µl | - | 104 | 106 | 102 | 104.00 | 2.00 | 0.00 |
| | | | + | 114 | 126 | 108 | 116.00 | 9.17 | 0.00 |
| Sodium azide | VII | 0.5 | - | 816 | 888 | 908 | 870.67 | 48.39 | 766.67 |
| 2-Aminoanthracene (Positive Control) | | 1 | + | 952 | 948 | 953 | 951.00 | 2.65 | 835.00 |
| Untreated Control | VIII | - | - | 100 | 98 | 114 | 104.00 | 8.72 | 0.00 |
| | | | + | 102 | 120 | 98 | 106.67 | 11.72 | -9.33 |

**TABLE NO.II (Contd.)**

**COLONY COUNTS OF HISTIDINE REVERTANTS IN *SALMONELLA***

Laboratory Test Item Code : TAS/002/017
Test System : *Salmonella typhimurium*

**Strain : TA 102** **Experiment No.: 2**

| Treatment | Group Number | Dose µg TOS/plate | Number of Revertant Colonies | | | | | | Induced Revertants |
|---|---|---|---|---|---|---|---|---|---|
| | | | S-9 | Individual Colony Counts | | | Mean | ± S.D. | |
| Test Item | I | 5000 | - | 310 | 324 | 300 | 311.33 | 12.06 | 38.67 |
| | | | + | 288 | 360 | 354 | 334.00 | 39.95 | 50.67 |
| | II | 1666.66 | - | 280 | 272 | 294 | 282.00 | 11.14 | 9.33 |
| | | | + | 310 | 322 | 298 | 310.00 | 12.00 | 26.67 |
| | III | 555.55 | - | 320 | 308 | 326 | 318.00 | 9.17 | 45.33 |
| | | | + | 326 | 330 | 318 | 324.67 | 6.11 | 41.33 |
| | IV | 185.18 | - | 294 | 280 | 288 | 287.33 | 7.02 | 14.67 |
| | | | + | 310 | 322 | 300 | 310.67 | 11.02 | 27.33 |
| | V | 61.72 | - | 280 | 288 | 296 | 288.00 | 8.00 | 15.33 |
| | | | + | 308 | 376 | 320 | 334.67 | 36.30 | 51.33 |
| Distilled Water (Solvent Control) | VI | 100 µl | - | 280 | 270 | 268 | 272.67 | 6.43 | 0.00 |
| | | | + | 290 | 272 | 288 | 283.33 | 9.87 | 0.00 |
| Mitomycin C | VII | 0.25 | - | 884 | 774 | 790 | 816.00 | 59.43 | 543.33 |
| Benzo(a)pyrene (Positive Control) | | 2.5 | + | 552 | 548 | 516 | 538.67 | 19.73 | 255.33 |
| Untreated Control | VIII | - | - | 230 | 240 | 218 | 229.33 | 11.02 | -43.33 |
| | | | + | 248 | 232 | 238 | 239.33 | 8.08 | -44.00 |

## TABLE NO.III

## SUMMARY DATA OF HISTIDINE REVERTANTS IN *SALMONELLA*

Laboratory Test Item Code : TAS/002/017
Test System : *Salmonella typhimurium*

### Experiment No.: 1

| Treatment | Dose µg TOS/ plate | Mean Number of Revertants and S.D. | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | TA1535 | | TA97a | | TA98 | | TA100 | | TA102 | |
| | | S-9 | Mean | ± SD | Mean | ± SD | Mean | ± SD | Mean | ± SD | Mean | ± SD |
| **Test Item** | 5000 | - | 12.67 | 1.53 | 84.00 | 3.00 | 31.33 | 3.06 | 84.33 | 6.11 | 304.00 | 21.17 |
| | | + | 13.67 | 1.53 | 90.67 | 2.52 | 31.00 | 3.46 | 84.33 | 2.52 | 314.00 | 37.04 |
| | 1666.66 | - | 12.00 | 1.00 | 76.33 | 2.52 | 26.67 | 2.52 | 121.33 | 22.03 | 291.33 | 15.53 |
| | | + | 14.00 | 1.00 | 83.00 | 1.00 | 29.00 | 2.65 | 119.67 | 3.79 | 297.33 | 9.02 |
| | 555.55 | - | 10.67 | 2.08 | 89.67 | 8.08 | 27.67 | 1.53 | 114.33 | 3.21 | 319.33 | 13.32 |
| | | + | 11.33 | 2.52 | 87.33 | 6.51 | 31.67 | 2.52 | 115.67 | 1.53 | 310.67 | 18.15 |
| | 185.18 | - | 11.00 | 0.00 | 84.00 | 2.00 | 29.33 | 4.04 | 123.00 | 7.81 | 287.33 | 7.02 |
| | | + | 13.00 | 1.00 | 86.00 | 3.00 | 30.67 | 2.52 | 126.00 | 6.93 | 293.33 | 4.62 |
| | 61.72 | - | 10.67 | 1.53 | 89.33 | 1.53 | 26.67 | 2.52 | 134.00 | 2.00 | 278.67 | 2.31 |
| | | + | 11.33 | 1.53 | 90.00 | 3.61 | 27.33 | 1.53 | 131.33 | 5.77 | 286.00 | 10.58 |
| Distilled Water | 100 | - | 9.00 | 1.00 | 75.33 | 5.13 | 24.00 | 1.00 | 102.67 | 5.03 | 248.67 | 9.02 |
| (Solvent Control) | µl | + | 11.00 | 1.00 | 83.33 | 4.04 | 25.00 | 1.73 | 108.67 | 7.57 | 264.00 | 6.00 |
| Sodium azide | 2 | - | 464.67 | 43.65 | - | - | - | - | - | - | - | - |
| | 0.5 | - | - | - | - | - | - | - | 904.00 | 17.78 | - | - |
| 9-Aminoacridine | 50 | - | - | - | 310.67 | 31.01 | - | - | - | - | - | - |
| 2-Nitrofluorene | 25 | - | - | - | - | - | 962.67 | 81.03 | - | - | - | - |
| Mitomycin C | 0.25 | - | - | - | - | - | - | - | - | - | 891.33 | 52.05 |
| Benzo(a)pyrene | 2.5 | + | - | - | 562.00 | 54.37 | - | - | - | - | 542.00 | 46.86 |
| 2 Aminoanthracene | 0.5 | + | 341.33 | 31.13 | - | - | - | - | - | - | - | - |
| | 5 | + | - | - | - | - | 1284.67 | 106.63 | - | - | - | - |
| | 1 | + | - | - | - | - | - | - | 942.67 | 50.65 | - | - |
| Positive Control | | | | | | | | | | | | |
| Untreated | - | - | 8.67 | 1.15 | 74.00 | 2.65 | 20.33 | 1.53 | 76.00 | 2.00 | 229.33 | 10.07 |
| Control | | + | 9.33 | 0.58 | 78.67 | 1.53 | 21.33 | 1.15 | 81.00 | 2.65 | 266.00 | 48.54 |

## TABLE NO.IV

## SUMMARY DATA OF HISTIDINE REVERTANTS IN *SALMONELLA*

Laboratory Test Item Code : TAS/002/017
Test System : *Salmonella typhimurium*

### Experiment No.: 2

| Treatment | Dose µg TOS/ plate | S-9 | Mean Number of Revertants and S.D. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | TA1535 | | TA97a | | TA98 | | TA100 | | TA102 | |
| | | | Mean | ± SD | Mean | ± SD | Mean | ± SD | Mean | ± SD | Mean | ± SD |
| Test Item | 5000 | - | 11.00 | 1.00 | 94.00 | 3.00 | 30.33 | 2.08 | 114.00 | 9.17 | 311.33 | 12.06 |
| | | + | 13.00 | 1.00 | 98.67 | 3.06 | 33.00 | 1.00 | 125.33 | 1.53 | 334.00 | 39.95 |
| | 1666.66 | - | 12.00 | 1.73 | 88.67 | 5.69 | 32.67 | 3.21 | 133.33 | 3.06 | 282.00 | 11.14 |
| | | + | 13.00 | 1.00 | 94.67 | 2.08 | 32.67 | 4.04 | 138.00 | 5.29 | 310.00 | 12.00 |
| | 555.55 | - | 10.00 | 1.73 | 88.00 | 1.00 | 27.00 | 1.00 | 124.00 | 4.00 | 318.00 | 9.17 |
| | | + | 12.33 | 1.53 | 91.00 | 2.00 | 29.00 | 1.00 | 126.00 | 4.00 | 324.67 | 6.11 |
| | 185.18 | - | 11.33 | 0.58 | 98.67 | 5.03 | 26.33 | 2.08 | 114.67 | 7.57 | 287.33 | 7.02 |
| | | + | 13.00 | 1.00 | 97.00 | 1.00 | 28.33 | 0.58 | 118.67 | 6.11 | 310.67 | 11.02 |
| | 61.72 | - | 10.67 | 1.53 | 87.67 | 6.51 | 27.67 | 3.21 | 110.00 | 10.58 | 288.00 | 8.00 |
| | | + | 11.33 | 1.53 | 89.67 | 1.15 | 29.00 | 2.65 | 111.33 | 11.72 | 334.67 | 36.30 |
| Distilled Water | 100 | - | 9.00 | 1.00 | 78.00 | 6.00 | 24.33 | 1.53 | 104.00 | 2.00 | 272.67 | 6.43 |
| (Solvent Control) | µl | + | 11.00 | 1.00 | 82.67 | 9.02 | 25.67 | 3.21 | 116.00 | 9.17 | 283.33 | 9.87 |
| Sodium azide | 2 | - | 444.67 | 31.39 | - | - | - | - | - | - | - | - |
| | 0.5 | - | - | - | - | - | - | - | 870.67 | 48.39 | - | - |
| 9-Aminoacridine | 50 | - | - | - | 310.00 | 19.08 | - | - | - | - | - | - |
| 2-Nitrofluorene | 25 | - | - | - | - | - | 903.33 | 28.02 | - | - | - | - |
| Mitomycin C | 0.25 | - | - | - | - | - | - | - | - | - | 816.00 | 59.43 |
| Benzo(a)pyrene | 2.5 | + | - | - | 539.33 | 43.10 | - | - | - | - | 538.67 | 19.73 |
| | 0.5 | + | 364.67 | 7.02 | - | - | - | - | - | - | - | - |
| 2 Aminoanthracene | 5 | + | - | - | - | - | 1355.33 | 64.63 | - | - | - | - |
| | 1 | + | - | - | - | - | - | - | 951.00 | 2.65 | - | - |
| Positive Control | | | | | | | | | | | | |
| Untreated | - | - | 8.33 | 0.58 | 73.67 | 1.53 | 21.33 | 1.15 | 104.00 | 8.72 | 229.33 | 11.02 |
| Control | | + | 9.00 | 1.00 | 74.00 | 2.00 | 23.33 | 0.58 | 106.67 | 11.72 | 239.33 | 8.08 |

## APPENDIX NO.I

## DOSE RANGE FINDING STUDY

Laboratory Test Item Code : TAS/002/017
Test System : *Salmonella typhimurium*

**Strain : TA 100**

| Treatment | Group Number | Dose µg TOS/ plate | Number of Revertant Colonies | | | | | | Induced Revertants |
|---|---|---|---|---|---|---|---|---|---|
| | | | S-9 | Individual Colony Counts | | | Mean | ± S.D. | |
| Test Item | I | 5000 | - | 120 | 144 | 146 | 136.67 | 14.47 | -13.33 |
| | | | + | 124 | 136 | 150 | 136.67 | 13.01 | -9.33 |
| | II | 1000 | - | 150 | 144 | 154 | 149.33 | 5.03 | -0.67 |
| | | | + | 152 | 140 | 150 | 147.33 | 6.43 | 1.33 |
| | III | 500 | - | 148 | 134 | 146 | 142.67 | 7.57 | -7.33 |
| | | | + | 158 | 160 | 146 | 154.67 | 7.57 | 8.67 |
| | IV | 100 | - | 170 | 180 | 176 | 175.33 | 5.03 | 25.33 |
| | | | + | 162 | 156 | 144 | 154.00 | 9.17 | 8.00 |
| | V | 50 | - | 158 | 162 | 158 | 159.33 | 2.31 | 9.33 |
| | | | + | 156 | 164 | 162 | 160.67 | 4.16 | 14.67 |
| | VI | 10 | - | 154 | 146 | 168 | 156.00 | 11.14 | 6.00 |
| | | | + | 160 | 168 | 172 | 166.67 | 6.11 | 20.67 |
| | VII | 5 | - | 144 | 168 | 152 | 154.67 | 12.22 | 4.67 |
| | | | + | 162 | 158 | 164 | 161.33 | 3.06 | 15.33 |
| | VIII | 1 | - | 170 | 164 | 158 | 164.00 | 6.00 | 14.00 |
| | | | + | 168 | 174 | 154 | 165.33 | 10.26 | 19.33 |
| Distilled Water (Solvent Control) | IX | 100 µl | - | 140 | 138 | 142 | 140.00 | 2.00 | 0.00 |
| | | | + | 148 | 152 | 138 | 146.00 | 7.21 | 0.00 |
| Untreated Control | X | Untreated Control | - | 124 | 128 | 130 | 127.33 | 3.06 | -22.67 |
| | | | + | 128 | 132 | 138 | 132.67 | 5.03 | -13.33 |

## APPENDIX NO.II

## HISTORICAL DATA OF NUMBER OF HISTIDINE REVERTANT COLONIES

### Historical Laboratory Control Data of the Negative Control (Untreated) and Positive Control(s) Without S9 (-S9) : 2015

| Untreated Control | TA 1535 | TA 97a | TA 98 | TA 100 | TA 102 |
|---|---|---|---|---|---|
| Mean | 8.91 | 80.24 | 21.67 | 82.95 | 223.33 |
| ±SD | 0.89 | 5.61 | 1.88 | 10.06 | 11.12 |
| Minimum | 7 | 72 | 19 | 71 | 204 |
| Maximum | 11 | 88 | 27 | 114 | 240 |

| Positive Control | TA 1535 | TA 97a | TA 98 | TA 100 | TA 102 |
|---|---|---|---|---|---|
| Concentration | Sodium azide | 9-Aminoacridine | 2-Nitrofluorene | Sodium azide | Mitomycin C |
| µg/plate | 2 | 50 | 25 | 0.5 | 0.25 |
| Mean | 422.33 | 282.43 | 951.90 | 820.57 | 824.38 |
| ±SD | 20.68 | 64.48 | 69.37 | 74.45 | 60.15 |
| Minimum | 380 | 170 | 842 | 696 | 720 |
| Maximum | 512 | 392 | 1072 | 928 | 942 |

### Historical Laboratory Control Data of the Negative Control (Untreated) and Positive Control(s) With S9 (+S9) : 2015

| Untreated Control | TA 1535 | TA 97a | TA 98 | TA 100 | TA 102 |
|---|---|---|---|---|---|
| Mean | 9.71 | 84.05 | 23.24 | 85.52 | 235.52 |
| ±SD | 1.02 | 6.06 | 1.67 | 11.83 | 22.70 |
| Minimum | 7 | 72 | 20 | 71 | 200 |
| Maximum | 12 | 93 | 27 | 120 | 322 |

| Positive Control | TA 1535 | TA 97a | TA 98 | TA 100 | TA 102 |
|---|---|---|---|---|---|
| Concentration | 2-Aminoanthracene | Benzo(a)pyrene | 2-Aminoanthracene | 2-Aminoanthracene | Benzo(a)pyrene |
| µg/plate | 0.5 | 2.5 | 5 | 1 | 2.5 |
| Mean | 325.65 | 492.38 | 1344.00 | 948.29 | 566.86 |
| ±SD | 20.62 | 76.74 | 87.22 | 130.67 | 94.25 |
| Minimum | 244 | 376 | 1172 | 720 | 384 |
| Maximum | 374 | 608 | 1512 | 1172 | 852 |

## APPENDIX NO.III

## CHARACTERIZATION OF *SALMONELLA* STRAINS

Test System : *Salmonella typhimurium*

| Genotype Characteristic | TA 1535 | TA 97a | TA 98 | TA 100 | TA 102 |
|---|---|---|---|---|---|
| **Histidine Requirement** | | | | | |
| Histidine - | - | - | - | - | - |
| Histidine + | + | + | + | + | + |
| **R - Factor** | | | | | |
| Ampicillin + | - | + | + | + | + |
| **rfa mutation** | | | | | |
| Zone of Inhibition (mm) | 10 | 11 | 12 | 12 | 14 |
| **UVrB - mutation** | | | | | |
| UV irradiated | - | - | - | - | - |
| UV non-irradiated | + | + | + | + | + |
| **PAQ1 plasmid** | | | | | |
| Ampicillin + Tetracycline | - | - | - | - | + |
| Mean Spontaneous Revertants | 9.33 | 90.33 | 28.00 | 105.33 | 220.33 |

# ANNEXURE - I

## Certificate of Analysis -
## Test Item (1 Page)
## and
## Information on Correction Factor
## (1 Page)

**Advanced Enzyme Technologies Ltd.,**
Plot No. A-61/62, MIDC, Malegaon, Tal. Sinnar, Dist. Nashik - 422 113,
Maharashtra, India. Tel.: +91-99701 00750 / +91-2551-230 044, Fax: +91-2551-230 816
Email: info@enzymeindia.com, Web.: www.enzymeindia.com

## QUALITY ASSURANCE DEPARTMENT

## CERTIFICATE OF ANALYSIS

**PRODUCT NAME : CHITOSANASE**
**BATCH NO. : 061434**
**MFG. DATE : JUNE,2014**
**EXPIRY DATE : MAY,2016**

### PROTOCOL OF ANALYSIS

| TEST | RESULT | LIMITS |
|---|---|---|
| **Description** | Light brown coloured powder; having characteristics odour. | Light brown to brown cloured powder with characteristic odour. |
| **Solubility** | Soluble in Water **: Complies** | Soluble in Water. |
| **Lead** | **: Complies** | Not more than 5 ppm |
| **Microbial Limit-**<br>**Total viable count**<br>**Total coliforms/g**<br>**Escherichia.coli/25g**<br>**Salmonellae/25g** | <br>**: Complies**<br>**: Complies**<br>**: Complies**<br>**: Complies** | <br>NMT 1 x $10^4$ cfu/g<br>Not more than 30<br>Negative by test<br>Negative by test |
| **Antimicrobial Activity** | Absent by test **: Complies** | Absent by test |
| **Chitosanase Activity** | 2,875 U/g | NLT 2,000 U/g |

**Remarks:** Sample **COMPLIES** as per Specifications.

**QA-CHEMIST**
**Date: October 28, 2014**

**MANAGER/QUALITY ASSURANCE**

**Advanced Enzyme Technologies Ltd.,**
Plot No. A-61/62, MIDC, Malegaon, Tal. Sinnar, Dist. Nashik - 422 113,
Maharashtra, India. Tel.: +91-99701 00750 / +91-2551-230 044, Fax: +91-2551-230 816
Email: info@enzymeindia.com, Web.: www.enzymeindia.com

Date: 03/11/14
Place: Sinnar

**TO WHOMSOEVER IT MAY CONCERN**

Relevant information on the test material is presented below.

Sample: Spray-dried unformulated concentrate of Chitosanase

Source: Bacillus subtilis subsp. subtilis

| Batch No. | 061434 |
|---|---|
| Ash (%) | 7.76 |
| Water (%) | 7.25 |
| TOS (%)* | 84.99 |
| Activity (U/g) | 2875 |
| U/mg TOS | 3.38 |
| Protein (%) | 61.69 |
| Heavy Metals | Complies** |
| Mycotoxins | Complies** |

* Calculated
**As per JECFA specifications (http://www.fao.org/ag/agn/jecfa-additives/docs/enzymes_en.htm)

The above information is provided for conducting toxicology studies.

**MANAGER–QUALITY**
Advanced Enzyme Technologies Ltd
Sinnar, Nasik-422 113
Maharashtra, India

# ANNEXURE - II

## Certificate of Approval
## (Good Laboratory Practice, GLP)
## (1 Page)

# NATIONAL GLP COMPLIANCE MONITORING AUTHORITY

## *GLP CERTIFICATE*

GLP Inspection was carried out at **Indian Institute of Toxicology, 32 A/1, Hadapsar Industrial Estate, Pune – 411013, Maharashtra, INDIA** in the following areas of expertise:

- **Toxicity studies**
  - Acute Studies (Oral, Parenteral, Dermal, Inhalation, Dermal Irritation/ Corrosion, Eye Irritation/ Corrosion, Skin Sensitization Test)
  - Sub-acute Studies
  - Chronic Studies
- **Mutagenicity Studies**
  - Bacterial Reverse Mutation Assay (Ames Test)
  - Mammalian Erythrocyte Micronucleus Test
  - *In vivo/ In Vitro* Mammalian Chromosome Aberration Test

Based on the Inspection Report and the follow-up actions taken by the test facility, it is confirmed that the test facility is capable of conducting the above-mentioned tests/studies in compliance with OECD Principles of Good Laboratory Practice (GLP) for the types of chemicals and in test systems as listed below respectively:

| | |
|---|---|
| Types of chemicals | : Industrial Chemicals, Pesticides, Pharmaceuticals, Veterinary Drugs, Cosmetics, Food additives and Feed additives |
| Test Systems | : Rat, Mice, Guinea pig, Rabbit, *Salmonella typhimurium,* tester strains viz. (TA 97a, TA 98, TA 100, TA 1535 & TA 102) |

This GLP Certificate is valid for a period of three years from April 16, 2013, subject to the condition that the test facility complies with the Terms & Conditions of the National GLP Compliance Monitoring Authority's Document Number GLP-101.

**Certificate No. : GLP/C-046**

**Issue Date : 10-04-2013**

**(VINITA SHARMA)**
**Head**
**National GLP Compliance Monitoring Authority**
**Department of Science & Technology**
**Technology Bhavan New Delhi-110016**

# ANNEXURE - III

## Summary of Amendment(s) to the Study Plan (1 Page)

## Summary of Amendment(s) to the Study Plan

| Amendment Number | Amendment |
|---|---|
| 1 | Study Completion Date finalized. |

30-JUN-2015
IIT STUDY NUMBER 18105

# INDIAN INSTITUTE OF TOXICOLOGY

भारतीय विषविज्ञान संस्था

FOUNDER DIRECTOR Late. DR. M. B. BHIDE (M.D.,F.C.A.I.)
Registered Office : "Kim"-2057, Sadashiv Peth, Pune - 411 030.
Correspondance Office : Flat No. 301, 401 "Usha-Shree" 90/124C, Erandawane, Gangote Street, Near Kamala Nehru Park, Pune - 411004.
Tel. Fax : (Testing Facility) : 020 - 2681 9961, 020 - 2681 9962 E-mail : iitoxicology@gmail.com

## FINAL REPORT

## IIT STUDY NUMBER 18105

## *IN VITRO* MAMMALIAN CHROMOSOME ABERRATION TEST OF CHITOSANASE IN HUMAN LYMPHOCYTES

**STUDY DIRECTOR**
**DR. (MRS.) R.P.DIGHE Ph.D.**

**TESTING FACILITY :**
**INDIAN INSTITUTE OF TOXICOLOGY**
**32 A/1, Hadapsar Industrial Estate,**
**Pune - 411 013.**

**SPONSOR'S REPRESENTATIVE**
**Mrs. Shilpa Risbud**

**SPONSOR ADDRESS :**
**ADVANCED ENZYME TECHNOLOGIES LTD.,**
**Sun Magnetica, 'A' wing, 5th Floor,**
**LIC Service Road,**
**Louiswadi, Thane (W) 400 604**
**Maharashtra, India.**

**REGULATORY REQUIREMENTS:**

OECD guidelines for Testing of Chemicals Section 4, *In vitro* Mammalian Chromosome Aberration Test in Human Lymphocytes, Test No. TG 473 Adopted on 26th September 2014.

**CONFIDENTIAL**

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## STATEMENT OF GLP COMPLIANCE

| | |
|---|---|
| Title of the Study | : *In Vitro* Mammalian Chromosome Aberration Test of Chitosanase in Human Lymphocytes |
| Study Number | : 18105 |
| Study Plan Number | : SPL/002/062 |
| Name of the Test item | : Chitosanase |

The study was conducted in accordance with the Good Laboratory Practice Principles as Published by the OECD in 1998, No 1 ENV/MC/CHEM(98)17.

| Mr. V.M.Bhide M.B.A. | | 30.06.2015 |
|---|---|---|
| Test Facility Management | Signature | Date |

## STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE

| | |
|---|---|
| Title of the Study | : *In Vitro* Mammalian Chromosome Aberration Test of Chitosanase in Human Lymphocytes |
| Study Number | : 18105 |
| Study Plan Number | : SPL/002/062 |
| Name of the Test item | : Chitosanase |

The study was conducted in compliance to the Good Laboratory Principles as Published by OECD in 1998, No 1 ENV/MC/CHEM(98)17.

The study was conducted in compliance to the written Study Plan approved by the Study Director and authorized by the Sponsor and Indian Institute of Toxicology Management and all applicable Standard Operating Procedures of Indian Institute of Toxicology, Pune.

All original Raw Data including documentation, slides generated during the course of the study, signed Study Plan, Study Plan Amendments and a copy of final report are archived at Indian Institute of Toxicology, Pune.

I accept responsibility for the conduct of the study and hereby declare that the study was conducted under my direction. This report is a complete, true and accurate record of the results obtained.

The sponsor is responsible for Good laboratory Practice (GLP) compliance for all test item information such as test item characterization, test item stability and Material Safety Data Sheet (MSDS) provided to test facility.

| Dr. (Mrs.) R.P.Dighe Ph.D. | | 30/06/2015 |
|---|---|---|
| Study Director | Signature | Date |

## STATEMENT OF QUALITY ASSURANCE UNIT

Title of the Study : *In Vitro* Mammalian Chromosome Aberration Test of Chitosanase in Human Lymphocytes

Study Number : 18105

Study Plan Number : SPL/002/062

Name of the Test item : Chitosanase

This study has been audited and the final report has been examined with respect to study plan, SOP and raw data. The report is true reflection of the raw data and that the study was conducted in compliance with the principles of GLP. The audits were carried out according to the applicable SOP's of Quality Assurance Unit of Indian Institute of Toxicology, Pune. The report is kept in the archives at Indian Institute of Toxicology, Pune.

Inspections were made by the Quality Assurance Unit of the Indian Institute of Toxicology for different phases of the study described in this report. The dates on which the inspections were made and the dates on which the findings were reported to the Study Director and to the facility Management are given below.

| Date(s) of Inspection | Phases Inspected | Date(s) findings reported to Study Director | Date(s) findings reported to Management |
|---|---|---|---|
| 16/12/2014 | Study Plan Review | 16/12/2014 | 16/12/2014 |
| 25/03/2015 | Pre Study Verification | 25/03/2015 | 25/03/2015 |
| 25/03/2015 | Amendment - 1 | 25/03/2015 | 25/03/2015 |
| 06/04/2015 | Setting of *In Vitro* Test System | 06/04/2015 | 06/04/2015 |
| 17/04/2015 | Harvesting of Cell Cultures | 17/04/2015 | 17/04/2015 |
| 18/04/2015 | Preparation of Slides | 18/04/2015 | 18/04/2015 |
| 21/05/2015 | Raw Data Audit | 21/05/2015 | 21/05/2015 |
| 22/05/2015 | Draft Report Audit | 22/05/2015 | 22/05/2015 |
| 30/05/2015 | Amendment - 2 | 30/05/2015 | 30/05/2015 |
| 29/06/2015 | Amendment - 3 | 29/06/2015 | 29/06/2015 |
| 30/06/2015 | Final Report Audit | 30/06/2015 | 30/06/2015 |

Dr. P.R.Tikhe Ph.D.

Quality Assurance Unit | Signature | 30.06.2015 Date

## PERSONNEL INVOLVED IN THE STUDY

| | |
|---|---|
| Study Director | : **Dr. R.P.Dighe Ph.D.** |
| Study Scientist | : **Mr. S.H.Oke M.S.** |
| Statistics | : **Mr. S.D.Nagpure B.Com.**<br>**Mr. D.K.Raut H.S.C.** |
| Quality Assurance Unit | : **Dr. P.R.Tikhe Ph.D.**<br>**Dr. R.M.Gosavi M.V.Sc.** |
| Report Preparation | : **Dr. R.P.Dighe Ph.D.**<br>**Mr. S.D.Nagpure B.Com.** |

# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| IIT | - | Indian Institute of Toxicology |
| GLP | - | Good Laboratory Practice |
| OECD | - | Organization for Economic Co-operation and Development |
| SOP | - | Standard Operating Procedure |
| mg | - | Milligram |
| µg | - | Microgram |
| µL | - | Microliter |
| °C | - | Degree Celsius |
| % | - | Percent |
| No. | - | Number |
| Nos. | - | Numbers |
| ml | - | Milliliter |
| S9 | - | Supernatant at 9000 g |
| EMS | - | Ethyl methanesulphonate |
| Conc. | - | Concentration |
| TOS | - | Total solids |

## SUMMARY AND CONCLUSION

The present study was conducted to evaluate the clastogenic potential of **Chitosanase**. Human Lymphocyte cultures were set and incubated at 37°C for 48 hours. The test item did not precipitate in Eagle's Minimum Essential Medium (MEM) at and up to 5 mg TOS/ml of culture. Therefore, cytotoxicity was conducted at test item concentration(s) of 5 mg TOS/ml of culture, 2.5 mg TOS/ml of culture, 1.25 mg TOS/ml of culture, 0.62 mg TOS/ml of culture and 0.31 mg TOS/ml of culture using mitotic index as an indicator of cytotoxicity.

Evaluation of mitotic index indicated that the test item did not inhibit mitotic activity at 5.0 mg TOS/ml of culture and therefore the test item was not cytotoxic at the concentration of 5 mg TOS/ml of culture. The reduction in mitotic activity was -8.75% to 14.57% at 5.0 mg TOS/ml of culture. Therefore, the highest concentration selected for the main study was 5 mg TOS/ml of culture and subsequent two lower concentrations selected were 2.5 mg TOS/ml of culture and 1.25 mg TOS/ml of culture.

Human Lymphocyte cultures were set and incubated at 37°C for 48 hours.

In first set, cultures with and without S9 were exposed to the test item for 4 hours at 5 mg TOS/ml of culture, 2.5 mg TOS/ml of culture and 1.25 mg TOS/ml of culture concentration followed by change of the medium.

Untreated cultures, cultures exposed to solvent distilled water and cultures exposed to positive control were run simultaneously.

In the second set, cultures with S9 were exposed to test item for 4 hours at 5 mg TOS/ml of culture, 2.5 mg TOS/ml of culture and 1.25 mg TOS/ml of culture followed by change of the medium.

Cultures without S9 were exposed to test item for 24 hours at 5 mg TOS/ml of culture, 2.5 mg TOS/ml of culture and 1.25 mg TOS/ml of culture.

Untreated cultures, cultures exposed to solvent and cultures exposed to positive control were run simultaneously.

All cultures were incubated at 37°C for 24 hours after exposure to the test item. Three hours prior to harvesting, cultures were treated with colchicine to arrest the cell cycle at metaphase.

Harvested cultures were centrifuged, treated with hypotonic solution, fixed with fixative (1:3 acetic acid : methanol) and centrifuged. The slides of the cells obtained were prepared and stained with Giemsa. Three hundred metaphases were scored at each test point for chromosomal break analysis.

The test item did not induce increased chromosomal aberrations at and up to the highest concentration of 5 mg TOS/ml of culture in comparison with solvent control.

Based on these results it is concluded that **Chitosanase** supplied by **Advanced Enzyme Technologies Ltd., Thane,** tested at and up to 5 mg TOS/ml of culture concentration did not induce chromosome aberrations in human lymphocyte cells *in vitro* and is therefore non-clastogenic in the presence and absence of microsomal enzymes (S-9 fraction).

**Dr. (Mrs.) R.P.Dighe**
**Study Director**

1) The results relate only to the items tested.
2) This report shall not be reproduced except in full, without the written approval of the laboratory.

## Schedule

Study Initiation Date : 16-12-2014

**Range Finding Study**

Experimental Starting Date : 06-04-2015

**Main Study**

Experimental Starting Date : 14-04-2015

Experiment Completion Date : 06-05-2015

**Study Completion Date : 30-06-2015**

Date of Reporting : 30-06-2015

## Archives

All raw data, study related documents and slides generated during the course of the study at Indian Institute of Toxicology, together with a copy of final report are lodged in the Indian Institute of Toxicology Archive.

Slides and records will be retained for a period of nine years from the date of issue of the final report. At the end of the nine years retention period, the sponsor will be contacted and an advice may be sought on the future requirements.

## Test Item Return

On completion of the study and submission of the final report, all unused samples of the test item was returned to the Sponsor.

## Animal Welfare

No animal was used in this study.

# OBJECTIVES

## Purpose

The purpose of this study was to evaluate the **Chitosanase** and/or its metabolites for their ability to induce chromosomal aberrations in mammalian cells *in vitro* in the presence and absence of mammalian microsomal enzymes (S9).

# MATERIALS AND METHODS

## Test Item

| | |
|---|---|
| Sponsor | : **Advanced Enzyme Technologies Ltd., Thane** |
| Laboratory Sponsor Code | : **SPN/002** |
| Test Item | : **Chitosanase** |
| Batch Number | : **061434** |
| Laboratory Test Item Code | : **TAS/002/017** |
| Manufacturing Date | : **June, 2014** |
| Expiry Date | : **May, 2016** |
| TOS (Total Solids) | : **84.99%** |
| Consistency | : **Solid powder** |
| Activity (Clinical Indication) | : **Biocatalyst for industry** |
| Safety Precautions | : Safety precautions included use of protective clothing, gloves, masks and eye protection (glasses). |
| Test Item Analysis | : Determination and documentation of the identity, strength, purity, stability and uniformity of the test item as defined in the Good Laboratory Practice (GLP) regulations, is the responsibility of the Sponsor. The Sponsor provided these test item characterization data (A Certificate of Analysis or equivalent) for review by the Study Director and inclusion in the final report. |
| Stability Data | : Information on file with the Sponsor. |
| Storage Condition | : Ambient temperature |
| Dose Preparation | : Test item was dissolved in distilled water at 50 mg TOS/ml of concentration. Two lower additional concentrations 25 mg TOS/ml and 12.5 mg TOS/ml were prepared by diluting the highest concentration using distilled water as vehicle. Actual preparation procedures are documented in the raw data. |
| Solubility Test | : Test item was dissolved in distilled water |
| Solvent | : Distilled water |

Disclaimer :

The above physiochemical data of test substance is supplied by the Sponsor. All responsibility with regards to the accuracy and authenticity of this information remains with the Sponsor. The test lab is not responsible for any variations with the batch number supplied.

**Test System**

**Mammalian cells *in vitro*:** Human Lymphocyte cell cultures

**Source:** A healthy human volunteer.

Healthy non smoking human volunteer, with no known illness or recent exposure to genotoxic agents was selected.
Human Lymphocyte cell cultures from whole blood were sampled in accordance to human ethical principles and regulations. All health records of volunteer are maintained in the raw data file.

**Characteristics:** Modal chromosome number (2n = 46)

**Rationale for selection of Human Lymphocytes cell cultures:**

1) In order to meet the regulatory requirement,
2) Availability of comprehensive background data at the test facility,
3) Widely used throughout the industry as preferred test system of choice in the genetic toxicological studies for assessment of *in-vitro* chromosomal aberrations,
4) Human Lymphocyte cell culture is the appropriate test system as the evaluation of data can be directly related to human beings.

## Study Design

### Culture Conditions:

Appropriate culture media and incubation conditions (culture vessels, $CO_2$ concentration, temperature and humidity) were used to maintain cultures. The cultures were monitored routinely for the absence of mycoplasma contamination and were not used if contaminated.
The normal cell cycle time for the cells and culture conditions used was pre-known.

Following culture media were used in the test:

Minimum essential medium (Hi media) supplemented with 10% Fetal Bovine Serum (FBS) to grow cell cultures, mitogen (phytohemagglutinin) to induce mitosis, anticoagulant (heparin) used to avoid clotting of blood, S9 mix (with metabolic activation) / phosphate buffer (without metabolic activation).

The following volumes were added to the culture vessel:

9.25 ml culture media containing 10% v/v FBS
0.4 ml heparinized whole blood
0.25 ml Phytohaemagglutinin
0.1 ml antibiotic solution
Total Volume (10 ml)

All the incubations were done at 37 °C.

Incubation Period: 72 hours

Number of cultures:

Duplicate cultures (with and without metabolic activation) were maintained at each concentration of test item, solvent control (negative control), untreated control and positive control. Total number of cultures maintained were 44 in number.

### Metabolic activation:

### Liver Homogenate S9 Fraction:

The Liver Homogenate S9 Fraction was available (preparation date 08-08-2014, batch number 13). The total proteins analysis was conducted and activity was evaluated and was found to be 41.1 mg/ml (acceptable range 35 - 45 mg/ml).
The S-9 mix was prepared immediately prior to its use in the assay. The microsomal reaction mixture (S-9 mix) contained the following components.
(D.M.Maron and B.N.Ames, (1983)

For 20 ml

| | |
|---|---|
| 1) S9 fraction | 2.00 ml |
| 2) 0.4 M MgCl2-0.65 M KCl salt solution | 0.40 ml |
| 3) 1.0 M G-6-P | 0.20 ml |
| 4) 0.4 mM NADP | 0.20 ml |
| 5) 0.2 M Phosphate buffer pH 7.4 | 17.20 ml |

**Experimental Design:**

Control Items:

Untreated controls with and without microsomal enzymes were used in the study.

Solvent controls (negative control): Distilled water with and without microsomal enzymes were employed in the study.

Positive Controls:

Ethyl methane sulphonate (EMS) at concentration of 120 µg per ml of culture was used without metabolic activation.

Benzo(a) pyrene at concentration of 0.2 µg per ml of culture was used with metabolic activation.

Justification for selection of Ethyl methane sulphonate (EMS) and Benzo(a)pyrene as positive control:

1) In order to meet the regulatory requirement of *In vitro* chromosomal aberration test;
2) Widely used in as positive control(s) in the *In vitro* chromosomal aberration test;
3) Availability of historical data on these positive control(s) at the facility.

**Cytotoxicity Study:**

Cytotoxicity was conducted at five different concentrations of the test item using mitotic index as an indicator of cytotoxicity. The highest concentration was 5 mg TOS/ml of culture. Four lower concentrations in the multiple of two were tested. Negative control (solvent used) was employed along with test concentrations.

**Selection of Concentrations for Main Study:**

Based on the results of the cytotoxicity study, the concentrations for main study were finalized. Evaluation of mitotic index indicated that the test item did not inhibit mitotic activity at 5.0 mg TOS/ml of culture and therefore the test item was not cytotoxic at the concentration of 5 mg TOS/ml of culture. The reduction in mitotic activity was 8.75% to 14.57% at 5.0 mg TOS/ml of culture. Therefore, the highest concentration selected for the main study was 5 mg TOS/ml of culture and subsequent two lower concentrations selected were 2.5 mg TOS/ml of culture and 1.25 mg TOS/ml of culture.

**Main Study:**

**Performance of the Test:**

Cultures of heparinized whole blood were induced with mitogen (PHA) were set at 37 °C for 48 hours. The set cultures were then exposed to 3 concentrations of the test item.

The cultures thus obtained were divided into two sets i.e. Set I and Set II.

Set I:

Cultures were exposed to the test item with and without S9 for 4 hours. Cells were washed followed by change of the medium.

Untreated cultures, cultures exposed to solvent and cultures exposed to positive control were run simultaneously.

Positive control included in set I was with metabolic activation for 4 hours of exposure.

Set II:

Cultures with S9 were exposed to test item for 4 hours followed by change of the medium.
Cultures without S9 were exposed to test item for 24 hours.

Untreated cultures, cultures exposed to solvent and cultures exposed to positive control were run simultaneously.

Positive control included in set II was without metabolic activation for 24 hours of exposure.

Set I and Set II:

All cultures were incubated at 37°C for 24 hours after the exposure of test item.

The cultures were treated with colchicine three hours prior to harvesting to arrest the cell cycle at metaphase and centrifuged at approximately 1200 rpm for 10 minutes. The harvested cultures were then treated with hypotonic solution followed by centrifugation and were fixed with fixative (1:3 acetic acid : methanol). The slides of the cells obtained were prepared and stained with Giemsa.

Scoring of slides:

The 300 cells were equally divided among the replicate(s) and 300 metaphases were scored at each test point for chromosomal break analysis.

**Type of Frequency of Aberrations and Observations:**

Cytotoxicity: Slides were scanned for cytotoxicity and evaluated for mitotic index. The metaphases were scored for chromatid and chromosome aberrations. In addition, gaps and abnormalities such as endoreduplication, polyploidy were recorded. Gaps, endoreduplication, polyploidy were not part of the final score of chromosome aberrations and were not considered as aberrations in the final scoring to conclude mutagenic potential of test item.

For each concentration, 300 metaphases were analyzed for structural aberrations.

For Mitotic Index 2000 cells were counted and percentage was calculated.

All the slides were coded before the analysis and decoded after the analysis.

Mitotic Index was calculated as follows:

$$\text{Mitotic Index} = \frac{\text{Total number of cells in division}}{\text{Total number of cells counted}} \times 100$$

**Data Analysis: (Table No.III, IV, V and VI)**

Total number of aberrations per cell, percent of cells aberrated was analysed for statistical significance using student 't' test.

**Acceptance Criteria: (Table No.III, IV, V, VI and Appendix No.I and II)**

The assay was considered valid for the following criteria:

1) The concurrent negative control data was considered acceptable for addition to the laboratory historical negative control data.
2) The concurrent positive control induced response that was compatible with those generated in historical positive control data and produced statistically significant increase compared with concurrent negative control.
3) Cell proliferation in the solvent control was fulfilled.
4) All experimental conditions tested unless one resulted in positive result

**Evaluation and Interpretation of Results:**

The test item was considered as positive in this assay as:

1) The proportion of cells with structural chromosome aberrations at one or more concentration exceeds the calculated normal range in both replicate experiments.
2) A statistical significant increase in the proportion of cells with structural chromosome aberrations (excluding gaps, Endoreduplication, polyploidy).

The test item was considered as negative in this assay as:

There was no requirement for verification of a clearly positive or negative response.

## RESULTS

### Mitotic Index (Table No.I and II)

**Cytotoxicity Assay:**

Set I :

Reduction in mitotic activity without and with metabolic activation at up to 5.0 mg TOS/ml of culture ranged between 0.19% and 8.75%.

Set II :

Reduction in mitotic activity without and with metabolic activation up to 5.0 mg TOS/ml of culture ranged between 0.95% and 14.57%.

### Chromosome Break Analysis

**Set I (4 hours exposure)**
**Without metabolic activation (Table No.III, Appendix No.I)**

The number of chromosome aberrations per cell was 0.01, 0.01 and 0.007 at 5 mg TOS/ml of culture, 2.5 mg TOS/ml of culture and 1.25 mg TOS/ml of culture concentrations. The number of chromosome aberrations per cell was 0.003 and 0.003 in untreated and solvent control group. The percent cells aberrated were 1.0, 1.0, 0.7 at 5 mg TOS/ml of culture, 2.5 mg TOS/ml of culture and 1.25 mg TOS/ml of culture concentrations. The percent cells aberrated were 0.3 and 0.3 in untreated, solvent control group. The aberrations induced were of chromatid type only. Gaps were not considered in calculation of total aberrations in cells.

### Chromosome Break Analysis

**Set I (4 hours exposure)**
**With metabolic activation (Table No.IV, Appendix No.I)**

The number of chromosome aberrations per cell was 0.013, 0.007 and 0.007 at 5 mg TOS/ml of culture, 2.5 mg TOS/ml of culture and 1.25 mg TOS/ml of culture concentrations. The number of chromosome aberrations per cell was 0.003 and 0.0 in untreated and solvent control group. It was 0.023 in positive control group. The percent cells aberrated were 1.3, 0.7, 0.7 at 5 mg TOS/ml of culture, 2.5 mg TOS/ml of culture and 1.25 mg TOS/ml of culture concentrations. The percent cells aberrated were 0.3 and 0.0 in untreated, solvent control group and 2.3 in the positive control group. The aberrations induced were of chromatid type only. Gaps were not considered in calculation of total aberrations in cells.

**Chromosome Break Analysis**

**Set II (24 hours exposure)**
**Without metabolic activation (Table No.V, Appendix No.II)**

The number of chromosome aberrations per cell was 0.007, 0.007 and 0.007 at 5 mg TOS/ml of culture, 2.5 mg TOS/ml of culture and 1.25 mg TOS/ml of culture concentrations. The number of chromosome aberrations per cell was 0.0 and 0.003 in untreated and solvent control group. It was 0.027 in positive control group. The percent cells aberrated were 0.7, 0.7, 0.7 at 5 mg TOS/ml of culture, 2.5 mg TOS/ml of culture and 1.25 mg TOS/ml of culture concentrations. The percent cells aberrated were 0.0 and 0.3 in untreated, solvent control group and 2.7 in the positive control group. The aberrations induced were of chromatid type only. Gaps were not considered in calculation of total aberrations in cells.

**Chromosome Break Analysis**

**Set II (4/24 hours exposure)**
**With metabolic activation (Table No.VI, Appendix No.II)**

The number of chromosome aberrations per cell was 0.01, 0.01 and 0.007 at 5 mg TOS/ml of culture, 2.5 mg TOS/ml of culture and 1.25 mg TOS/ml of culture concentrations. The number of chromosome aberrations per cell was 0.0 and 0.0 in untreated and solvent control group. The percent cells aberrated were 1.0, 1.0, 0.7 at 5 mg TOS/ml of culture, 2.5 mg TOS/ml of culture and 1.25 mg TOS/ml of culture concentrations. The percent cells aberrated were 0.0 and 0.0 in untreated, solvent control group. The aberrations induced were of chromatid type only. Gaps were not considered in calculation of total aberrations in cells.

**Numerical aberrations (Appendix No.III)**

Set I and Set II (4 hours exposure) and Set II (24 hours exposure):
The test item induced polyploidy in the cultures exposed to 1.25 mg TOS/ml of culture with metabolic activation and at 2.5 mg TOS/ml of culture without and with metabolic activation.

## REGULATORY REFERENCES

1) OECD guidelines for Testing of Chemicals Section 4, *In vitro* Mammalian Chromosome Aberration Test in Human Lymphocytes, Test No. TG 473 Adopted on 26th September 2014.

2) OECD Principles of Good Laboratory Practice: Document # 1, ENV/MC/CHEM (98)17.

3) Recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines for Laboratory Animal Facility published in The Gazette of India, December 15, 1998.

4) Requirements and Guidelines for permission to Import and/or Manufacture of New Drugs for the sale or to undertake clinical trails. Schedule "Y", Drugs and Cosmetics (IInd Amendment) Rules, Ministry of Health and Family Welfare, Government of India, January 20, 2005.

5) Revised methods for the Salmonella mutagenicity test, D.M.Maron and B.N.Ames, Mutation Research, 113 pg 207 (1983).

## TABLE NO.I

## MITOTIC INDEX OF SET I

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Duration of Exposure : 4 hours

| Group Number | Concentration mg TOSS per ml of culture | S9 | Total Number of cells | Number of Dividing cells | Mitotic Index (%) | Reduction in Mitotic Index (%) |
|---|---|---|---|---|---|---|
| I | 0 | - | 2005 | 108 | 5.39 | - |
| | | + | 2005 | 106 | 5.29 | - |
| II | 10.0 µl | - | 2005 | 104 | 5.20 | 3.52 |
| | | + | 2005 | 103 | 5.14 | 2.83 |
| III | 0.31 | - | 2004 | 100 | 4.99 | 4.04 |
| | | + | 2009 | 104 | 5.18 | 0.78 |
| IV | 0.62 | - | 2005 | 106 | 5.29 | 1.85 |
| | | + | 2000 | 102 | 5.10 | 0.78 |
| V | 1.25 | - | 2002 | 103 | 5.14 | 4.64 |
| | | + | 2007 | 103 | 5.13 | 0.19 |
| VI | 2.5 | - | 2005 | 105 | 5.24 | 2.78 |
| | | + | 2002 | 105 | 5.24 | 1.94 |
| VII | 5.0 | - | 2000 | 102 | 5.10 | 1.92 |
| | | + | 2005 | 94 | 4.69 | 8.75 |

- = Without metabolic activation
+ = With metabolic activation

## TABLE NO.II

## MITOTIC INDEX OF SET II

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Duration of Exposure : 24 hours (-S9)
4 hours (+S9)

| Group Number | Concentration mg TOSS per ml of culture | S9 | Total Number of cells | Number of Dividing cells | Mitotic Index (%) | Reduction in Mitotic Index (%) |
|---|---|---|---|---|---|---|
| I | 0 | - | 2003 | 106 | 5.29 | - |
| | | + | 2000 | 91 | 4.55 | - |
| II | 10.0 µl | - | 2002 | 106 | 5.29 | 0.0 |
| | | + | 2005 | 110 | 5.49 | + 20.66 |
| III | 0.31 | - | 2002 | 112 | 5.59 | + 5.67 |
| | | + | 2002 | 106 | 5.29 | 3.64 |
| IV | 0.62 | - | 2005 | 108 | 5.39 | + 1.89 |
| | | + | 2005 | 102 | 5.09 | 8.20 |
| V | 1.25 | - | 2001 | 102 | 5.10 | 3.59 |
| | | + | 2000 | 105 | 5.25 | 4.37 |
| VI | 2.5 | - | 2005 | 105 | 5.24 | 0.95 |
| | | + | 2005 | 102 | 5.09 | 7.28 |
| VII | 5.0 | - | 2000 | 112 | 5.10 | 3.59 |
| | | + | 2005 | 94 | 4.69 | 14.57 |

- = Without metabolic activation
+ = With metabolic activation

## TABLE NO.III

## SUMMARY OF CHROMOSOME BREAK ANALYSIS OF SET I WITHOUT METABOLIC ACTIVATION

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Duration of Exposure : 4 hours

Metabolic Activation : - S9

| Group Number | Treatment | Concentration mg TOS per ml of culture | Total number of metaphases analyzed | Total No. of Aberrations | Type of aberrations | | | Total no. of cells with aberration | Aberration per cell | Percent Cells with Aberration |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Gap• | Chromatid | Chromosome | | | |
| I | Untreated control | 0.0 | 300 | 1 | 1 | 1B | 0 | 1 | 0.003 | 0.3 |
| II | Solvent (Negative) control | 10 µl | 300 | 1 | 1 | 1B | 0 | 1 | 0.003 | 0.3 |
| III | Test Item | 5.0 | 300 | 3 | 2 | 3B | 0 | 3 | 0.01 | 1.0 |
| IV | Test Item | 2.5 | 300 | 3 | 2 | 3B | 0 | 3 | 0.01 | 1.0 |
| V | Test Item | 1.25 | 300 | 2 | 2 | 2B | 0 | 2 | 0.007 | 0.7 |
| VI | Positive ○ control | - | - | - | - | - | - | - | - | - |

• = Not considered for calculations of aberrations.
○ = Positive control in this set is not included.
- S9 = Without metabolic activation

## TABLE NO.IV

## SUMMARY OF CHROMOSOME BREAK ANALYSIS OF SET I WITH METABOLIC ACTIVATION

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Duration of Exposure : 4 hours

Metabolic Activation : + S9

| Group Number | Treatment | Concentration mg TOS per ml of culture | Total number of metaphases analyzed | Total No. of Aberrations | Type of aberrations | | | Total no. of cells with aberration | Aberration per cell | Percent Cells with Aberration |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Gap• | Chromatid | Chromosome | | | |
| I | Untreated control | 0.0 | 300 | 1 | 1 | 1B | 0 | 1 | 0.003 | 0.3 |
| II | Solvent (Negative) control | 10 µl | 300 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| III | Test Item | 5.0 | 300 | 4 | 3 | 4B | 0 | 4 | 0.013 | 1.3 |
| IV | | 2.5 | 300 | 2 | 1 | 2 | 0 | 2 | 0.007 | 0.7 |
| V | | 1.25 | 300 | 2 | 1 | 2B | 0 | 2 | 0.007 | 0.7 |
| VI | Positive control | 0.2 µg | 300 | 7 | 3 | 7B | 0 | 7 | 0.023 | 2.3** |

• = Not considered for calculations of aberrations.
** = Significant at 99% level of confidence ($p \leq 0.01$)
+S9 = With metabolic activation

## TABLE NO.V

## SUMMARY OF CHROMOSOME BREAK ANALYSIS OF SET II WITHOUT METABOLIC ACTIVATION

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Duration of Exposure : 24 hours

Metabolic Activation : - S9

| Group Number | Treatment | Concentration mg TOS per ml of culture | Total number of metaphases analyzed | Total No. of Aberrations | Type of aberrations | | | Total no. of cells with aberration | Aberration per cell | Percent Cells with Aberration |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Gap• | Chromatid | Chromosome | | | |
| I | Untreated control | 0.0 | 300 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| II | Solvent (Negative) control | 10 µl | 300 | 1 | 1 | 1B | 0 | 1 | 0.003 | 0.3 |
| III | Test Item | 5.0 | 300 | 2 | 1 | 2B | 0 | 2 | 0.007 | 0.7 |
| IV | Test Item | 2.5 | 300 | 2 | 2 | 2B | 0 | 2 | 0.007 | 0.7 |
| V | Test Item | 1.25 | 300 | 2 | 1 | 2B | 0 | 2 | 0.007 | 0.7 |
| VI | Positive control | 120 µg | 300 | 8 | 4 | 8B | 0 | 8 | 0.027 | 2.7* |

• = Not considered for calculations of aberrations.
* = Significant at 95% level of confidence ($p \leq 0.05$)
- S9 = Without metabolic activation

## TABLE NO.VI

## SUMMARY OF CHROMOSOME BREAK ANALYSIS OF SET II WITH METABOLIC ACTIVATION

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Duration of Exposure : 4/24 hours

Metabolic Activation : + S9

| Group Number | Treatment | Concentration mg TOS per ml of culture | Total number of metaphases analyzed | Total No. of aberrations | Type of aberrations | | | Total no. of cells with aberration | Aberration per cell | Percent Cells with Aberration |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Gap• | Chromatid | Chromosome | | | |
| I | Untreated control | 0.0 | 300 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| II | Solvent (Negative) control | 10 µl | 300 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| III | Test Item | 5.0 | 300 | 3 | 2 | 3B | 0 | 3 | 0.01 | 1.0 |
| IV | Test Item | 2.5 | 300 | 3 | 2 | 3B | 0 | 3 | 0.01 | 1.0 |
| V | Test Item | 1.25 | 300 | 2 | 1 | 2B | 0 | 2 | 0.007 | 0.7 |
| VI | Positive ○ control | - | - | - | - | - | - | - | - | - |

• = Not considered for calculations of aberrations.
○ = Positive control in this set is not included.
+S9 = With metabolic activation

## APPENDIX NO.I

## CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET I

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Conc. : 0.0 mg TOS/ml of culture (Untreated Control)

Duration of Exposure : 4 hours

Metabolic activation : - S9

| Culture | | 1 | | | 2 | | | Total |
|---|---|---|---|---|---|---|---|---|
| Slide Number | | 1.1 | 1.2 | ◘ | 2.1 | 2.2 | ◘ | 4 |
| Total Number of Metaphases Analyzed | | 95 | 55 | ◘ | 82 | 68 | ◘ | 300 |
| Type of Aberrations | Gap | 1 | 0 | ◘ | 0 | 0 | ◘ | 1 |
| | Chromatid Break/Fragment | 1B | 0 | ◘ | 0 | 0 | ◘ | 1B |
| | Chromosome | 0 | 0 | ◘ | 0 | 0 | ◘ | 0 |
| Total Number of Aberrations | | 1 | 0 | ◘ | 0 | 0 | ◘ | 1 |
| Total Number of cells Aberrated | | 1 | 0 | ◘ | 0 | 0 | ◘ | 1 |
| Aberrations per cell | | 0.01 | 0 | ◘ | 0 | 0 | ◘ | 0.003 |

◘ = Slide not scored.

- S9 = Without metabolic activation

**APPENDIX NO.I (Contd.)**

## CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET I

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Conc. : 500 µl/ml of culture Solvent Control (Negative Control)

Duration of Exposure : 4 hours

Metabolic activation : - S9

| Culture | | 5 | | | 6 | | | Total |
|---|---|---|---|---|---|---|---|---|
| Slide Number | | 5.1 | ◘ | ◘ | 6.1 | ◘ | ◘ | 2 |
| Total Number of Metaphases Analyzed | | 150 | ◘ | ◘ | 150 | ◘ | ◘ | 300 |
| Type of Aberrations | Gap | 0 | ◘ | ◘ | 1 | ◘ | ◘ | 1 |
| | Chromatid Break/Fragment | 0 | ◘ | ◘ | 1B | ◘ | ◘ | 1B |
| | Chromosome | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| Total Number of Aberrations | | 0 | ◘ | ◘ | 1 | ◘ | ◘ | 1 |
| Total Number of cells Aberrated | | 0 | ◘ | ◘ | 1 | ◘ | ◘ | 1 |
| Aberrations per cell | | 0 | ◘ | ◘ | 0.007 | ◘ | ◘ | 0.003 |

◘ = Slide not scored.
- S9 = Without metabolic activation

## APPENDIX NO.I (Contd.)

## CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET I

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Conc. : 1.25 mg TOS/ml of culture

Duration of Exposure : 4 hours

Metabolic activation : - S9

| Culture | | 13 | | | 14 | | | Total |
|---|---|---|---|---|---|---|---|---|
| Slide Number | | 13.1 | ◘ | ◘ | 14.1 | ◘ | ◘ | 2 |
| Total Number of Metaphases Analyzed | | 150 | ◘ | ◘ | 150 | ◘ | ◘ | 300 |
| Type of Aberrations | Gap | 1 | ◘ | ◘ | 1 | ◘ | ◘ | 2 |
| | Chromatid Break/Fragment | 1B | ◘ | ◘ | 1B | ◘ | ◘ | 2B |
| | Chromosome | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| Total Number of Aberrations | | 1 | ◘ | ◘ | 1 | ◘ | ◘ | 2 |
| Total Number of cells Aberrated | | 1 | ◘ | ◘ | 1 | ◘ | ◘ | 2 |
| Aberrations per cell | | 0.007 | ◘ | ◘ | 0.007 | ◘ | ◘ | 0.007 |

◘ = Slide not scored.
- S9 = Without metabolic activation

## APPENDIX NO.I (Contd.)

## CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET I

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Conc. : 2.5 mg TOS/ml of culture

Duration of Exposure : 4 hours

Metabolic activation : - S9

| Culture | | 17 | | | 18 | | | Total |
|---|---|---|---|---|---|---|---|---|
| Slide Number | | 17.1 | ◘ | ◘ | 18.1 | ◘ | ◘ | 2 |
| Total Number of Metaphases Analyzed | | 150 | ◘ | ◘ | 150 | ◘ | ◘ | 300 |
| Type of Aberrations | Gap | 1 | ◘ | ◘ | 1 | ◘ | ◘ | 2 |
| | Chromatid Break/Fragment | 2B | ◘ | ◘ | 1B | ◘ | ◘ | 3B |
| | Chromosome | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| Total Number of Aberrations | | 2 | ◘ | ◘ | 1 | ◘ | ◘ | 3 |
| Total Number of cells Aberrated | | 2 | ◘ | ◘ | 1 | ◘ | ◘ | 3 |
| Aberrations per cell | | 0.013 | ◘ | ◘ | 0.007 | ◘ | ◘ | 0.01 |

◘ = Slide not scored.
B = Break
- S9 = Without metabolic activation

## APPENDIX NO.I (Contd.)

## CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET I

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Conc. : 5.0 mg TOS/ml of culture

Duration of Exposure : 4 hours    Metabolic activation : - S9

| Culture | | 21 | | | 22 | | | Total |
|---|---|---|---|---|---|---|---|---|
| Slide Number | | 21.1 | ◘ | ◘ | 22.2 | ◘ | ◘ | 2 |
| Total Number of Metaphases Analyzed | | 150 | ◘ | ◘ | 150 | ◘ | ◘ | 300 |
| Type of Aberrations | Gap | 1 | ◘ | ◘ | 1 | ◘ | ◘ | 2 |
| | Chromatid Break/Fragment | 1B | ◘ | ◘ | 2B | ◘ | ◘ | 3B |
| | Chromosome | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| Total Number of Aberrations | | 1 | ◘ | ◘ | 2 | ◘ | ◘ | 3 |
| Total Number of cells Aberrated | | 1 | ◘ | ◘ | 2 | ◘ | ◘ | 3 |
| Aberrations per cell | | 0.007 | ◘ | ◘ | 0.013 | ◘ | ◘ | 0.01 |

◘ = Slide not scored.
- S9 = Without metabolic activation

## APPENDIX NO.I (Contd.)

## CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET I

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Conc. : 0.0 mg TOS/ml of culture (Untreated control)

Duration of Exposure : 4 hours

Metabolic activation : + S9

| Culture | | 3 | | | 4 | | | Total |
|---|---|---|---|---|---|---|---|---|
| Slide Number | | 3.1 | ◘ | ◘ | 4.1 | ◘ | ◘ | 2 |
| Total Number of Metaphases Analyzed | | 150 | ◘ | ◘ | 150 | ◘ | ◘ | 300 |
| Type of Aberrations | Gap | 1 | ◘ | ◘ | 0 | ◘ | ◘ | 1 |
| | Chromatid Break/Fragment | 1B | ◘ | ◘ | 0 | ◘ | ◘ | 1B |
| | Chromosome | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| Total Number of Aberrations | | 1 | ◘ | ◘ | 0 | ◘ | ◘ | 1 |
| Total Number of cells Aberrated | | 1 | ◘ | ◘ | 0 | ◘ | ◘ | 1 |
| Aberrations per cell | | 0.007 | ◘ | ◘ | 0 | ◘ | ◘ | 0.003 |

◘ = Slide not scored.
+S9 = With metabolic activation

## APPENDIX NO.I (Contd.)

## CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET I

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Conc. : 500 µl/ml of culture Solvent Control (Negative Control)

Duration of Exposure : 4 hours    Metabolic activation : + S9

| Culture | | 7 | | | 8 | | | Total |
|---|---|---|---|---|---|---|---|---|
| Slide Number | | 7.1 | ◘ | ◘ | 8.1 | ◘ | ◘ | 2 |
| Total Number of Metaphases Analyzed | | 150 | ◘ | ◘ | 150 | ◘ | ◘ | 300 |
| Type of Aberrations | Gap | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| | Chromatid Break/Fragment | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| | Chromosome | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| Total Number of Aberrations | | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| Total Number of cells Aberrated | | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| Aberrations per cell | | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |

◘ = Slide not scored.
+S9 = With metabolic activation

**APPENDIX NO.I (Contd.)**

## CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET I

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Conc. : 0.2 µg/ml of Culture (Benzo[a] pyrene)

Duration of Exposure : 4 hours | Metabolic activation : + S9

| Culture | | 9 | | | 10 | | | Total |
|---|---|---|---|---|---|---|---|---|
| Slide Number | | 9.1 | ◘ | ◘ | 10.1 | ◘ | ◘ | 2 |
| Total Number of Metaphases Analyzed | | 150 | ◘ | ◘ | 150 | ◘ | ◘ | 300 |
| Type of Aberrations | Gap | 0 | ◘ | ◘ | 3 | ◘ | ◘ | 3 |
| | Chromatid Break/Fragment | 2B | ◘ | ◘ | 5B | ◘ | ◘ | 7B |
| | Chromosome | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| Total Number of Aberrations | | 2 | ◘ | ◘ | 5 | ◘ | ◘ | 7 |
| Total Number of cells Aberrated | | 2 | ◘ | ◘ | 5 | ◘ | ◘ | 7 |
| Aberrations per cell | | 0.013 | ◘ | ◘ | 0.033 | ◘ | ◘ | 0.023 |

◘ = Slide not scored.
B =Break
+S9 = With metabolic activation

## APPENDIX NO.I (Contd.)

## CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET I

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Conc. : 1.25 mg TOS/ml of culture

Duration of Exposure : 4 hours

Metabolic activation : + S9

| Culture | | 15 | | | 16 | | | Total |
|---|---|---|---|---|---|---|---|---|
| Slide Number | | 15.1 | ◘ | ◘ | 16.1 | ◘ | ◘ | 2 |
| Total Number of Metaphases Analyzed | | 150 | ◘ | ◘ | 150 | ◘ | ◘ | 300 |
| Type Of Aberrations | Gap | 0 | ◘ | ◘ | 1 | ◘ | ◘ | 1 |
| | Chromatid Break/Fragment | 1B | ◘ | ◘ | 1B | ◘ | ◘ | 2B |
| | Chromosome | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| Total Number of Aberrations | | 1 | ◘ | ◘ | 1 | ◘ | ◘ | 2 |
| Total Number of cells Aberrated | | 1 | ◘ | ◘ | 1 | ◘ | ◘ | 2 |
| Aberrations per cell | | 0.007 | ◘ | ◘ | 0.007 | ◘ | ◘ | 0.007 |

◘ = Slide not scored.
+S9 = With metabolic activation

## APPENDIX NO.I (Contd.)

## CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET I

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Conc. : 2.5 mg TOS/ml of culture

Duration of Exposure : 4 hours

Metabolic activation : + S9

| Culture | | 19 | | | 20 | | | Total |
|---|---|---|---|---|---|---|---|---|
| Slide Number | | 19.1 | ◘ | ◘ | 20.1 | ◘ | ◘ | 2 |
| Total Number of Metaphases Analyzed | | 150 | ◘ | ◘ | 150 | ◘ | ◘ | 300 |
| Type of Aberrations | Gap | 1 | ◘ | ◘ | 0 | ◘ | ◘ | 1 |
| | Chromatid Break/Fragment | 1B | ◘ | ◘ | 1B | ◘ | ◘ | 2B |
| | Chromosome | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| Total Number of Aberrations | | 1 | ◘ | ◘ | 1 | ◘ | ◘ | 2 |
| Total Number of cells Aberrated | | 1 | ◘ | ◘ | 1 | ◘ | ◘ | 2 |
| Aberrations per cell | | 0.007 | ◘ | ◘ | 0.007 | ◘ | ◘ | 0.007 |

◘ = Slide not scored.
B = Break
+S9 = With metabolic activation

## APPENDIX NO.I (Contd.)

## CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET I

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Conc. : 5.0 mg TOS/ml of culture

Duration of Exposure : 4 hours

Metabolic activation : + S9

| Culture | | 23 | | | 24 | | | Total |
|---|---|---|---|---|---|---|---|---|
| Slide Number | | 23.1 | 23.2 | ◘ | 24.1 | 24.2 | ◘ | 4 |
| Total Number of Metaphases Analyzed | | 36 | 114 | ◘ | 105 | 45 | ◘ | 300 |
| Type of Aberrations | Gap | 1 | 1 | ◘ | 0 | 1 | ◘ | 3 |
| | Chromatid Break/Fragment | 1B | 1B | ◘ | 1B | 1B | ◘ | 4B |
| | Chromosome | 0 | 0 | ◘ | 0 | 0 | ◘ | 0 |
| Total Number of Aberrations | | 1 | 1 | ◘ | 1 | 1 | ◘ | 4 |
| Total Number of cells Aberrated | | 1 | 1 | ◘ | 1 | 1 | ◘ | 4 |
| Aberrations per cell | | 0.028 | 0.009 | ◘ | 0.009 | 0.022 | ◘ | 0.013 |

◘ = Slide not scored.
+S9 = With metabolic activation

# APPENDIX NO.II

## CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET II

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Conc. : 1.25 mg TOS/ml of culture

Duration of Exposure : 24 hours

Metabolic activation : - S9

| Culture | | 25 | | | 26 | | | Total |
|---|---|---|---|---|---|---|---|---|
| Slide Number | | 25.1 | ◘ | ◘ | 26.1 | 26.2 | ◘ | 3 |
| Total Number of Metaphases Analyzed | | 150 | ◘ | ◘ | 100 | 50 | ◘ | 300 |
| Type of Aberrations | Gap | 0 | ◘ | ◘ | 1 | 0 | ◘ | 1 |
| | Chromatid Break/Fragment | 1B | ◘ | ◘ | 1B | 0 | ◘ | 2B |
| | Chromosome | 0 | ◘ | ◘ | 0 | 0 | ◘ | 0 |
| Total Number of Aberrations | | 1 | ◘ | ◘ | 1 | 0 | ◘ | 2 |
| Total Number of cells Aberrated | | 1 | ◘ | ◘ | 1 | 0 | ◘ | 2 |
| Aberrations per cell | | 0.007 | ◘ | ◘ | 0.01 | 0 | ◘ | 0.007 |

◘ = Slide not scored.
B = Break
- S9 = Without metabolic activation

## APPENDIX NO.II (Contd.)

## CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET II

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Conc. : 2.5 mg TOS/ml of culture

Duration of Exposure : 24 hours

Metabolic activation : - S9

| Culture | | 29 | | | 30 | | | Total |
|---|---|---|---|---|---|---|---|---|
| Slide Number | | 29.1 | 29.2 | ◘ | 30.1 | 30.2 | ◘ | 4 |
| Total Number of Metaphases Analyzed | | 125 | 25 | ◘ | 125 | 25 | ◘ | 300 |
| Type of Aberrations | Gap | 1 | 0 | ◘ | 1 | 0 | ◘ | 2 |
| | Chromatid Break/Fragment | 1B | 0 | ◘ | 1B | 0 | ◘ | 2B |
| | Chromosome | 0 | 0 | ◘ | 0 | 0 | ◘ | 0 |
| Total Number of Aberrations | | 1 | 0 | ◘ | 1 | 0 | ◘ | 2 |
| Total Number of cells Aberrated | | 1 | 0 | ◘ | 1 | 0 | ◘ | 2 |
| Aberrations per cell | | 0.008 | 0 | ◘ | 0.008 | 0 | ◘ | 0.007 |

◘ = Slide not scored.
B = Break
- S9 = Without metabolic activation

## APPENDIX NO.II (Contd.)

## CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET II

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Conc. : 5.0 mg TOS/ml of culture

Duration of Exposure : 24 hours

Metabolic activation : - S9

| Culture | | 33 | | | 34 | | | Total |
|---|---|---|---|---|---|---|---|---|
| Slide Number | | 33.1 | 33.2 | ◘ | 34.1 | 34.2 | ◘ | 4 |
| Total Number of Metaphases Analyzed | | 95 | 55 | ◘ | 22 | 128 | ◘ | 300 |
| Type of Aberrations | Gap | 0 | 0 | ◘ | 0 | 1 | ◘ | 1 |
| | Chromatid Break/Fragment | 0 | 0 | ◘ | 1B | 1B | ◘ | 2B |
| | Chromosome | 0 | 0 | ◘ | 0 | 0 | ◘ | 0 |
| Total Number of Aberrations | | 0 | 0 | ◘ | 1 | 1 | ◘ | 2 |
| Total Number of cells Aberrated | | 0 | 0 | ◘ | 1 | 1 | ◘ | 2 |
| Aberrations per cell | | 0 | 0 | ◘ | 0.045 | 0.008 | ◘ | 0.007 |

◘ = Slide not scored.
B = Break
- S9 = Without metabolic activation

## APPENDIX NO.II (Contd.)

## CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET II

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Conc. : 0.0 mg TOS/ml of culture (Untreated Control)

Duration of Exposure : 24 hours

Metabolic activation : - S9

| Culture | | 37 | | | 38 | | | Total |
|---|---|---|---|---|---|---|---|---|
| Slide Number | | 37.1 | ▣ | ▣ | 38.2 | ▣ | ▣ | 2 |
| Total Number of Metaphases Analyzed | | 150 | ▣ | ▣ | 150 | ▣ | ▣ | 300 |
| Type of Aberrations | Gap | 0 | ▣ | ▣ | 0 | ▣ | ▣ | 0 |
| | Chromatid Break/Fragment | 0 | ▣ | ▣ | 0 | ▣ | ▣ | 0 |
| | Chromosome | 0 | ▣ | ▣ | 0 | ▣ | ▣ | 0 |
| Total Number of Aberrations | | 0 | ▣ | ▣ | 0 | ▣ | ▣ | 0 |
| Total Number of cells Aberrated | | 0 | ▣ | ▣ | 0 | ▣ | ▣ | 0 |
| Aberrations per cell | | 0 | ▣ | ▣ | 0 | ▣ | ▣ | 0 |

▣ = Slide not scored.
- S9 = Without metabolic activation

## APPENDIX NO.II (Contd.)

## CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET II

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Conc. : 500 μl/ml of culture Solvent Control (Negative Control)

Duration of Exposure : 24 hours

Metabolic activation : - S9

| Culture | | 41 | | | 42 | | | Total |
|---|---|---|---|---|---|---|---|---|
| Slide Number | | 41.2 | ◘ | ◘ | 42.1 | ◘ | ◘ | 2 |
| Total Number of Metaphases Analyzed | | 150 | ◘ | ◘ | 150 | ◘ | ◘ | 300 |
| Type of Aberrations | Gap | 1 | ◘ | ◘ | 0 | ◘ | ◘ | 1 |
| | Chromatid Break/Fragment | 1B | ◘ | ◘ | 0 | ◘ | ◘ | 1B |
| | Chromosome | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| Total Number of Aberrations | | 1 | ◘ | ◘ | 0 | ◘ | ◘ | 1 |
| Total Number of cells Aberrated | | 1 | ◘ | ◘ | 0 | ◘ | ◘ | 1 |
| Aberrations per cell | | 0.007 | ◘ | ◘ | 0 | ◘ | ◘ | 0.003 |

◘ = Slide not scored.
- S9 = Without metabolic activation

**APPENDIX NO.II (Contd.)**

## CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET II

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Conc. : 120 µg/ml of Culture (Ethyl Methane Sulphonate)

Duration of Exposure : 24 hours

Metabolic activation : - S9

| Culture | | 11 | | | 12 | | | Total |
|---|---|---|---|---|---|---|---|---|
| Slide Number | | 11.1 | 11.2 | 11.3 | 12.1 | 12.3 | ◘ | 5 |
| Total Number of Metaphases Analyzed | | 34 | 66 | 50 | 117 | 33 | ◘ | 300 |
| Type of Aberrations | Gap | 0 | 1 | 0 | 3 | 0 | ◘ | 4 |
| | Chromatid Break/Fragment | 0 | 3B | 1B | 4B | 0 | ◘ | 8B |
| | Chromosome | 0 | 0 | 0 | 0 | 0 | ◘ | 0 |
| Total Number of Aberrations | | 0 | 3 | 1 | 4 | 0 | ◘ | 8 |
| Total Number of cells Aberrated | | 0 | 3 | 1 | 4 | 0 | ◘ | 8 |
| Aberrations per cell | | 0 | 0.045 | 0.02 | 0.034 | 0 | ◘ | 0.027 |

◘ = Slide not scored.
B = Break
- S9 = Without metabolic activation

## APPENDIX NO.II (Contd.)

## CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET II

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Conc. : 1.25 mg TOS/ml of culture

Duration of Exposure : 4/24 hours

Metabolic activation : + S9

| Culture | | 27 | | | 28 | | | Total |
|---|---|---|---|---|---|---|---|---|
| Slide Number | | 27.1 | 27.2 | ◘ | 28.1 | 28.2 | ◘ | 4 |
| Total Number of Metaphases Analyzed | | 75 | 75 | ◘ | 117 | 33 | ◘ | 300 |
| Type of Aberrations | Gap | 1 | 0 | ◘ | 0 | 0 | ◘ | 1 |
| | Chromatid Break/Fragment | 1B | 0 | ◘ | 1B | 0 | ◘ | 2B |
| | Chromosome | 0 | 0 | ◘ | 0 | 0 | ◘ | 0 |
| Total Number of Aberrations | | 1 | 0 | ◘ | 1 | 0 | ◘ | 2 |
| Total Number of cells Aberrated | | 1 | 0 | ◘ | 1 | 0 | ◘ | 2 |
| Aberrations per cell | | 0.013 | 0 | ◘ | 0.008 | 0 | ◘ | 0.007 |

◘ = Slide not scored.
+S9 = With metabolic activation

## APPENDIX NO.II (Contd.)

## CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET II

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Conc. : 2.5 mg TOS/ml of culture

Duration of Exposure : 4/24 hours

Metabolic activation : + S9

| Culture | | 31 | | | 32 | | | Total |
|---|---|---|---|---|---|---|---|---|
| Slide Number | | 31.1 | 31.2 | ◘ | 32.1 | ◘ | ◘ | 3 |
| Total Number of Metaphases Analyzed | | 32 | 118 | ◘ | 150 | ◘ | ◘ | 300 |
| Type Of Aberrations | Gap | 0 | 0 | ◘ | 2 | ◘ | ◘ | 2 |
| | Chromatid Break/Fragment | 0 | 1B | ◘ | 2B | ◘ | ◘ | 3B |
| | Chromosome | 0 | 0 | ◘ | 0 | ◘ | ◘ | 0 |
| Total Number of Aberrations | | 0 | 1 | ◘ | 2 | ◘ | ◘ | 3 |
| Total Number of cells Aberrated | | 0 | 1 | ◘ | 2 | ◘ | ◘ | 3 |
| Aberrations per cell | | 0 | 0.008 | ◘ | 0.013 | ◘ | ◘ | 0.01 |

◘ = Slide not scored.
+S9 = With metabolic activation

**APPENDIX NO.II (Contd.)**

## CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET II

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Conc. : 5.0 mg TOS/ml of culture

Duration of Exposure : 4/24 hours

Metabolic activation : + S9

| Culture | | 35 | | | 36 | | | Total |
|---|---|---|---|---|---|---|---|---|
| Slide Number | | 35.1 | 35.2 | ◘ | 36.2 | 36.3 | ◘ | 4 |
| Total Number of Metaphases Analyzed | | 100 | 50 | ◘ | 98 | 52 | ◘ | 300 |
| Type Of Aberrations | Gap | 1 | 0 | ◘ | 1 | 0 | ◘ | 2 |
| | Chromatid Break/Fragment | 2B | 0 | ◘ | 1B | 0 | ◘ | 3B |
| | Chromosome | 0 | 0 | ◘ | 0 | 0 | ◘ | 0 |
| Total Number of Aberrations | | 2 | 0 | ◘ | 1 | 0 | ◘ | 3 |
| Total Number of cells Aberrated | | 2 | 0 | ◘ | 1 | 0 | ◘ | 3 |
| Aberrations per cell | | 0.02 | 0 | ◘ | 0.01 | 0 | ◘ | 0.01 |

◘ = Slide not scored.
B = Break
+S9 = With metabolic activation

**APPENDIX NO.II (Contd.)**

## CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET II

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Conc. : 0.0 mg TOS/ml of culture (Untreated Control)

Duration of Exposure : 4/24 hours

Metabolic activation : + S9

| Culture | | 39 | | | 40 | | | Total |
|---|---|---|---|---|---|---|---|---|
| Slide Number | | 39.1 | ◘ | ◘ | 40.1 | ◘ | ◘ | 2 |
| Total Number of Metaphases Analyzed | | 150 | ◘ | ◘ | 150 | ◘ | ◘ | 300 |
| Type Of Aberrations | Gap | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| | Chromatid Break/Fragment | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| | Chromosome | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| Total Number of Aberrations | | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| Total Number of cells Aberrated | | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| Aberrations per cell | | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |

◘ = Slide not scored.
+S9 = With metabolic activation

## APPENDIX NO.II (Contd.)

## CHROMOSOME ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET II

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

Conc. : 500 µl/ml of culture Solvent Control (Negative Control)

Duration of Exposure : 4/24 hours | Metabolic activation : + S9

| Culture | | 43 | | | 44 | | | Total |
|---|---|---|---|---|---|---|---|---|
| Slide Number | | 43.1 | ◘ | ◘ | 44.1 | ◘ | ◘ | 2 |
| Total Number of Metaphases Analyzed | | 150 | ◘ | ◘ | 150 | ◘ | ◘ | 300 |
| Type of Aberrations | Gap | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| | Chromatid Break/Fragment | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| | Chromosome | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| Total Number of Aberrations | | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| Total Number of cells Aberrated | | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |
| Aberrations per cell | | 0 | ◘ | ◘ | 0 | ◘ | ◘ | 0 |

◘ = Slide not scored.
+S9 = With metabolic activation

## APPENDIX NO.III

## NUMERICAL ABERRATIONS IN HUMAN LYMPHOCYTES *IN VITRO* OF SET I AND SET II

Laboratory Test Item Code : TAS/002/017
Test System : Human Lymphocyte Culture

| Group Number | Concentration mg TOS/ml of culture | S9 | Duration of Exposure (hours) | | | |
|---|---|---|---|---|---|---|
| | | | 4 P | 4 E | 4 / 24 P | 4 / 24 E |
| I | Untreated | - | 0 | 0 | 0 | 0 |
| | Control | + | 0 | 0 | 0 | 0 |
| II | Solvent (Negative) | - | 0 | 0 | 0 | 0 |
| | Control | + | 0 | 0 | 0 | 0 |
| III | 5.0 | - | 0 | 0 | 0 | 0 |
| | | + | 0 | 0 | 0 | 0 |
| IV | 2.5 | - | 1 | 0 | 0 | 0 |
| | | + | 1 | 0 | 0 | 0 |
| V | 1.25 | - | 0 | 0 | 0 | 0 |
| | | + | 1 | 0 | 0 | 0 |

- = Without metabolic activation
+ = With metabolic activation
P : Polyploidy
E : Endoreduplication

## APPENDIX NO.IV

## HISTORICAL DATA OF *IN VITRO* MAMMALIAN CHROMOSOME ABERRATION TEST

Test System : Human Lymphocytes

Duration of Exposure : 4 hours Metabolic Activation : - S9

Parameter: Percent Cells with Aberration

| Study Number | Untreated Control | Positive Control | Vehicle/Solvent (Negative Control) | | | Historical Data Made On |
|---|---|---|---|---|---|---|
| | | | Distilled Water | DMSO | DMF | |
| | Mean | Mean | Mean | Mean | Mean | |
| Average | 0.00 | - | 0.13 | 0.22 | 1.00 | 31-10-2013 |
| 17431 | 0.0 | - | 0.0 | - | - | 27-02-2014 |
| 17436 | 0.0 | - | 0.5 | - | - | 04-04-2014 |
| 17783 | 0.0 | - | 0.3 | - | - | 17-11-2014 |
| 17775 | 0.0 | - | 0.3 | - | - | 28-11-2014 |
| 17825 | 0.0 | - | 0.3 | - | - | 05-02-2015 |
| Average | 0.00 | - | 0.26 | 0.22 | 1.00 | |

## APPENDIX NO.IV (Contd.)

## HISTORICAL DATA OF *IN VITRO* MAMMALIAN CHROMOSOME ABERRATION TEST

Test System : Human Lymphocytes

Duration of Exposure : 4 hours

Metabolic Activation : + S9

Parameter: Percent Cells with Aberration

| Study Number | Untreated Control | Positive Control | Vehicle/Solvent (Negative Control) | | | Historical Data Made On |
|---|---|---|---|---|---|---|
| | | | Distilled Water | DMSO | DMF | |
| | Mean | Mean | Mean | Mean | Mean | |
| Average | 0.00 | 4.30 | 0.07 | 0.24 | 1.00 | 31-10-2013 |
| 17431 | 0.0 | 3.0 | 0.0 | - | - | 27-02-2014 |
| 17436 | 0.0 | 3.5 | 0.5 | - | - | 04-04-2014 |
| 17783 | 0.0 | 2.7 | 0.7 | - | - | 17-11-2014 |
| 17775 | 0.3 | 3.0 | 0.3 | - | - | 28-11-2014 |
| 17825 | 0.0 | 3.0 | 0.3 | - | - | 05-02-2015 |
| Average | 0.05 | 3.25 | 0.31 | 0.24 | 1.00 | |

## APPENDIX NO.IV (Contd.)

## HISTORICAL DATA OF *IN VITRO* MAMMALIAN CHROMOSOME ABERRATION TEST

Test System : Human Lymphocytes

Duration of Exposure : 24 hours Metabolic Activation : - S9

Parameter: Percent Cells with Aberration

| Study Number | Untreated Control | Positive Control | Vehicle/Solvent (Negative Control) | | | Historical Data Made On |
|---|---|---|---|---|---|---|
| | | | Distilled Water | DMSO | DMF | |
| | Mean | Mean | Mean | Mean | Mean | |
| Average | 0.13 | 4.66 | 0.13 | 0.32 | 1.00 | 31-10-2013 |
| 17431 | 0.0 | 3.0 | 0.0 | - | - | 27-02-2014 |
| 17436 | 0.0 | 3.5 | 0.5 | - | - | 04-04-2014 |
| 17783 | 0.0 | 3.0 | 0.3 | - | - | 17-11-2014 |
| 17775 | 0.0 | 3.0 | 0.0 | - | - | 28-11-2014 |
| 17825 | 0.0 | 2.7 | 0.3 | - | - | 05-02-2015 |
| Average | 0.02 | 3.31 | 0.21 | 0.32 | 1.00 | |

## APPENDIX NO.IV (Contd.)

## HISTORICAL DATA OF *IN VITRO* MAMMALIAN CHROMOSOME ABERRATION TEST

Test System : Human Lymphocytes

Duration of Exposure : 4 hours Metabolic Activation : + S9

Parameter: Percent Cells with Aberration

| Study Number | Untreated Control | Positive Control | Vehicle/Solvent (Negative Control) | | | Historical Data Made On |
|---|---|---|---|---|---|---|
| | | | Distilled Water | DMSO | DMF | |
| | Mean | Mean | Mean | Mean | Mean | |
| Average | 0.01 | - | 0.13 | 0.28 | 1.00 | 31-10-2013 |
| 17431 | 0.0 | - | 0.0 | - | - | 27-02-2014 |
| 17436 | 0.0 | - | 0.5 | - | - | 04-04-2014 |
| 17783 | 0.0 | - | 0.7 | - | - | 17-11-2014 |
| 17775 | 0.0 | - | 0.7 | - | - | 28-11-2014 |
| 17825 | 0.3 | - | 0.3 | - | - | 05-02-2015 |
| Average | 0.05 | - | 0.39 | 0.28 | 1.00 | |

# ANNEXURE - I

## Certificate of Analysis - Test Item (1 Page) and Information on Correction Factor (1 Page)

**Advanced Enzyme Technologies Ltd.,**
Plot No. A-61/62, MIDC, Malegaon, Tal. Sinnar, Dist. Nashik - 422 113,
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Email: info@enzymeindia.com, Web.: www.enzymeindia.com

## QUALITY ASSURANCE DEPARTMENT

## CERTIFICATE OF ANALYSIS

**PRODUCT NAME : CHITOSANASE**
**BATCH NO. : 061434**
**MFG. DATE : JUNE,2014**
**EXPIRY DATE : MAY,2016**

### PROTOCOL OF ANALYSIS

| TEST | RESULT | LIMITS |
|---|---|---|
| **Description** | Light brown coloured powder; having characteristics odour. | Light brown to brown cloured powder with characteristic odour. |
| **Solubility** | Soluble in Water **: Complies** | Soluble in Water. |
| **Lead** | **: Complies** | Not more than 5 ppm |
| **Microbial Limit-**<br>**Total viable count**<br>**Total coliforms/g**<br>**Escherichia.coli/25g**<br>**Salmonellae/25g** | <br>**: Complies**<br>**: Complies**<br>**: Complies**<br>**: Complies** | <br>NMT $1 \times 10^4$ cfu/g<br>Not more than 30<br>Negative by test<br>Negative by test |
| **Antimicrobial Activity** | Absent by test **: Complies** | Absent by test |
| **Chitosanase Activity** | 2,875 U/g | NLT 2,000 U/g |

**Remarks:** Sample **COMPLIES** as per Specifications.

**QA-CHEMIST** **MANAGER-QUALITY ASSURANCE**
**Date: October 28, 2014**

**Advanced Enzyme Technologies Ltd.,**
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Date: 03/11/14
Place: Sinnar

**TO WHOMSOEVER IT MAY CONCERN**

Relevant information on the test material is presented below.

Sample: Spray-dried unformulated concentrate of Chitosanase

Source: Bacillus subtilis subsp. subtilis

| **Batch No.** | **061434** |
|---|---|
| Ash (%) | 7.76 |
| Water (%) | 7.25 |
| TOS (%)* | 84.99 |
| Activity (U/g) | 2875 |
| U/mg TOS | 3.38 |
| Protein (%) | 61.69 |
| Heavy Metals | Complies** |
| Mycotoxins | Complies** |

* Calculated
**As per JECFA specifications (http://www.fao.org/ag/agn/jecfa-additives/docs/enzymes_en.htm)

The above information is provided for conducting toxicology studies.

**MANAGER–QUALITY**
Advanced Enzyme Technologies Ltd
Sinnar, Nasik-422 113
Maharashtra, India

# ANNEXURE - II

## Certificate of Approval
## (Good Laboratory Practice, GLP)
## (1 Page)

# NATIONAL GLP COMPLIANCE MONITORING AUTHORITY

## *GLP CERTIFICATE*

GLP Inspection was carried out at **Indian Institute of Toxicology, 32 A/1, Hadapsar Industrial Estate, Pune – 411013, Maharashtra, INDIA** in the following areas of expertise:

- **Toxicity studies**
  - Acute Studies (Oral, Parenteral, Dermal, Inhalation, Dermal Irritation/ Corrosion, Eye Irritation/ Corrosion, Skin Sensitization Test)
  - Sub-acute Studies
  - Chronic Studies
- **Mutagenicity Studies**
  - Bacterial Reverse Mutation Assay (Ames Test)
  - Mammalian Erythrocyte Micronucleus Test
  - *In vivo/ In Vitro* Mammalian Chromosome Aberration Test

Based on the Inspection Report and the follow-up actions taken by the test facility, it is confirmed that the test facility is capable of conducting the above-mentioned tests/studies in compliance with OECD Principles of Good Laboratory Practice (GLP) for the types of chemicals and in test systems as listed below respectively:

| | |
|---|---|
| Types of chemicals | : Industrial Chemicals, Pesticides, Pharmaceuticals, Veterinary Drugs, Cosmetics, Food additives and Feed additives |
| Test Systems | : Rat, Mice, Guinea pig, Rabbit, *Salmonella typhimurium*, tester strains viz. (TA 97a, TA 98, TA 100, TA 1535 & TA 102) |

This GLP Certificate is valid for a period of three years from April 16, 2013, subject to the condition that the test facility complies with the Terms & Conditions of the National GLP Compliance Monitoring Authority's Document Number GLP-101.

**Certificate No. : GLP/C-046**

**Issue Date : 10-04-2013**

**(VINITA SHARMA)**
**Head**
**National GLP Compliance Monitoring Authority**
**Department of Science & Technology**
**Technology Bhavan New Delhi-110016**

# ANNEXURE - III

## Summary of Amendment(s) to the Study Plan (1 Page)

# Summary of Amendment(s) to the Study Plan

| Amendment Number | Amendment |
|---|---|
| 1 | The Study Schedule dates finalized. |
| 2 | Experimental Completion Date added and Study Completion Date revised. |
| 3 | Study Completion Date finalized. |

| DEPARTMENT OF HEALTH AND HUMAN SERVICES<br>Food and Drug Administration<br>**GENERALLY RECOGNIZED AS SAFE (*GRAS*) NOTICE** (Subpart E of Part 170) | Form Approved: OMB No. 0910-0342; Expiration Date: 07/31/2022<br>*(See last page for OMB Statement)* |
|---|---|

**FDA USE ONLY**

| GRN NUMBER | DATE OF RECEIPT |
|---|---|
| 000991 | Dec 15, 2020 |
| ESTIMATED DAILY INTAKE | INTENDED USE FOR INTERNET |
| NAME FOR INTERNET | |
| KEYWORDS | |

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration,5001 Campus Drive, College Park, MD 20740-3835.

## SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No.________ ☐ Supplement to GRN No. ________

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3 Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd)*: ________

4 For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd)*: ________

## SECTION B – INFORMATION ABOUT THE NOTIFIER

**1a. Notifier**

| Name of Contact Person | Position or Title |
|---|---|
| ANIL KUMAR GUPTA, PHD | VP - RESEARCH & DEVELOPMENT |

Organization *(if applicable)*: ADVANCED ENZYME TECHNOLOGIES LTD.

Mailing Address *(number and street)*: MAGNETICA LIC SERVICE ROAD, LOUISWADI

| City | State or Province | Zip Code/Postal Code | Country |
|---|---|---|---|
| THANE | MAHARASHTRA | 400604 | India |

| Telephone Number | Fax Number | E-Mail Address |
|---|---|---|
| +91 22 25830284 | | ANIL@ADVANCEDENZYMES.COM |

**1b. Agent or Attorney *(if applicable)***

| Name of Contact Person | Position or Title |
|---|---|
| KEVIN GILLIES | MEMBER |

Organization *(if applicable)*: KEVIN O. GILLIES CONSULTING SERVICES, LLC

Mailing Address *(number and street)*: 1759 GRAPE ST.

| City | State or Province | Zip Code/Postal Code | Country |
|---|---|---|---|
| Denver | CO | 80220 | US |

| Telephone Number | Fax Number | E-Mail Address |
|---|---|---|
| 001-816-590-9836 | | KEVIN.O.GILLIES@GMAIL.COM |

## SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

CHITOSANASE FROM BACILLUS SUBTILIS CSSC

2. Submission Format: *(Check appropriate box(es))*

☒ Electronic Submission Gateway

☐ Electronic files on physical media

☐ Paper

If applicable give number and type of physical media ______

3. For paper submissions only:

Number of volumes ______

Total number of pages ______

4. Does this submission incorporate any information in CFSAN's files? *(Check one)*

☐ Yes *(Proceed to Item 5)* ☒ No *(Proceed to Item 6)*

5. The submission incorporates information from a previous submission to FDA as indicated below *(Check all that apply)*

☐ a) GRAS Notice No. GRN ______

☐ b) GRAS Affirmation Petition No. GRP ______

☐ c) Food Additive Petition No. FAP ______

☐ d) Food Master File No. FMF ______

☐ e) Other or Additional *(describe or enter information as above)* ______

6. Statutory basis for conclusions of GRAS status *(Check one)*

☒ Scientific procedures *(21 CFR 170.30(a) and (b))* ☐ Experience based on common use in food *(21 CFR 170.30(a) and (c))*

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8))

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

## SECTION D – INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

Chitosanase from Bacillus subtilis CSSC is intended to be used as a processing aid in chitosan hydrolysis. The resultant hydrolysate Water Soluble Chitosan (WSC) is intended to be used as a preservative in the food industry, more specifically in the preservation of shrimp.

Chitosanase produces enzymatically hydrolyzed WSC, which inter alia, preserves properties (e.g. moisture content, color, texture etc.) of shrimp during storage and improves product shelf life. WSC can substitute for conventional chemical preservatives in this application. (Yu Guang-li et al. 1996; Chouljenko et al. 2016).

h Ch ( C ) h l d d f OS/k l

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

*(Check one)*

☐ Yes ☒ No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☐ No , you ask us to exclude trade secrets from the information FDA will send to FSIS.

## SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

*(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)*

☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).

☒ PART 3 of a GRAS notice: Dietary exposure (170.235).

☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).

☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).

☒ PART 6 of a GRAS notice: Narrative (170.250).

☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

**Other Information**

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☐ Yes ☒ No

Did you include this other information in the list of attachments?

☐ Yes ☐ No

## SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that ADVANCED ENZYME TECHNOLOGIES LTD.
*(name of notifier)*

has concluded that the intended use*(s)* of Chitosanase from Bacillus subtilis CSSC
*(name of notified substance)*

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. ADVANCED ENZYME TECHNOLOGIES LTD. *(name of notifier)* agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.

Advanced Enzyme Technologies, Ltd., 4880 Murrieta Street, Chino, CA 91710
*(address of notifier or other location)*

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance.The notifying party certifies that the information provided herein is accurate and complete to the best or his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

| 3. Signature of Responsible Official, Agent, or Attorney | Printed Name and Title | Date (mm/dd/yyyy) |
|---|---|---|
| Kevin O. Gillies Digitally signed by Kevin O. Gillies Date: 2020.12.14 16:09:31 -07'00' | KEVIN O. GILLIES, AGENT | 12/14/2020 |

## SECTION G – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

| Attachment Number | Attachment Name | Folder Location (select from menu) (Page Number(s) for paper Copy Only) |
|---|---|---|
| | Form3667.pdf | Administrative |
| | AnnexA4_CAtest_Chitosanase.pdf | GRAS Notice |
| | AnnexA3_AMESTest_Chitosanase.pdf | GRAS Notice |
| | AnnexA2_90daystoxicity_FinalReport.pdf | GRAS Notice |
| | AnnexA1_AcuteOralToxicity.pdf | GRAS Notice |
| | Chitosanase_GRASNOTICE_12142020_Final.pdf | GRAS Notice |
| | Chitosanase_GRASNOTICE_12142020_Final.pdf | Administrative |

**OMB Statement:** Public reporting burden for this collection of information is estimated to average 170 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services,Food and Drug Administration, Office of Chief Information Officer, PRAStaff@fda.hhs.gov. (Please do NOT return the form to this address.). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.

## Viebrock, Lauren

**From:** Kevin Gillies <kevin.o.gillies@gmail.com>
**Sent:** Wednesday, October 13, 2021 12:55 PM
**To:** Viebrock, Lauren
**Cc:** Ankit Rathi; Rasika Rathi
**Subject:** [EXTERNAL] Re: Acknowledgement of filing of GRAS Notice No. GRN 000991

CAUTION: This email originated from outside of the organization. Do not click links or open attachments unless you recognize the sender and know the content is safe.

Dear Dr. VieBrock,
I am writing on behalf of my client Advanced Enzyme Technologies to inform you that I am closing my consulting business and we would like further communication on GRN 000991 to go to:

Name: Ankit Rathi

Email: ankit@specialtyenzymes.com

Phone: +1-909-613-1660

Mail address: 4880 Murrieta Street, Chino, CA – 91710.

Thank you.

Best,

Kevin Gillies

Kevin O. Gillies Consulting Services, LLC
1759 Grape St.
Denver, CO 80220

Tel:+1 816 590 9836

On Wed, Jun 9, 2021 at 9:47 AM Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov> wrote:

> Dear Mr. Gillies,
>
> Attached to this email, please find the acknowledgement letter for filing of the GRAS notice you submitted to our office, which has been designated as GRAS Notice No. GRN 000989.

Please let me know if you have any questions.

Regards,

Lauren VieBrock

## Viebrock, Lauren

**From:** Anil Gupta <anil@advancedenzymes.com>
**Sent:** Wednesday, January 19, 2022 12:23 AM
**To:** Viebrock, Lauren
**Subject:** [EXTERNAL] Re: GRAS Notice No. GRN 000991

CAUTION: This email originated from outside of the organization. Do not click links or open attachments unless you recognize the sender and know the content is safe.

Dear Lauren,
Thank you for contacting us. I would like to confirm that you can communicate with Mr Ankit rathi for future communication as suggested byMr Kevin.
Best Regards
Dr Anil Gupta

On Fri, Jan 14, 2022 at 12:54 AM Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov> wrote:

Dear Dr. Gupta,

The agent representing Advanced Enzymes for GRN 991 (chitosanase enzyme preparation), Mr. Kevin Gillies, informed us that he has closed his business and provided a contact for communications pertaining to this GRAS notice as Ankit Rathi affiliated with Specialty Enzymes. As you are the representative of Advanced Enzymes listed on the GRAS notice, I want to confirm that you would like us to communicate with Mr. Rathi on future communications for GRN 991. Thank you.

Regards,

Lauren VieBrock

**Lauren VieBrock**
*Regulatory Review Scientist/Microbiology Reviewer*

**Center for Food Safety and Applied Nutrition**
**Office of Food Additive Safety**
**U.S. Food and Drug Administration**
Tel: 301-796-7454
lauren.viebrock@fda.hhs.gov

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Advanced Enzyme Technologies Ltd
Sun Magnetica, 'A' wing, 5th floor
LIC Service Road, Louiswadi, Thane (W) 400604, India
Tel +91-22-41703200

Disclaimer :: This email and any files transmitted with it are confidential and intended solely for the use of the individual or entity to whom they are addressed. If you are not the named addressee you should not disseminate, distribute or copy this e-mail. Please notify the sender immediately by e-mail if you have received this e-mail by mistake and delete this e-mail from your system. Please note that any views or opinions presented in this email are solely those of the author and do not necessarily represent those of the company. The recipient should check this email and any attachments for the presence of viruses. The company accepts no liability for any damage caused by any virus transmitted by this email.

## Viebrock, Lauren

**From:** Ankit Rathi <Ankit@specialtyenzymes.com>
**Sent:** Friday, June 3, 2022 1:50 PM
**To:** Viebrock, Lauren
**Subject:** [EXTERNAL] RE: GRN 000991 Questions
**Attachments:** 2022-06-03 AET response to FDA questions.zip; GRN 991 Questions_Response_June 2022.pdf

CAUTION: This email originated from outside of the organization. Do not click links or open attachments unless you recognize the sender and know the content is safe.

Dear Dr. Viebrock,

Happy Friday! Hope you are doing well. In response to your email dated May 20, 2022, we have attached a PDF file with the responses, and Annexes B – K (11 PDF files) in a zip file. Please let me know if you have any questions. I can be reached by email, or by phone at 909-613-1660.

Have a great weekend!

Regards,
Ankit Rathi

---

**From:** Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
**Sent:** Friday, May 20, 2022 9:49 PM
**To:** Ankit Rathi <Ankit@specialtyenzymes.com>
**Subject:** [BULK] GRN 000991 Questions
**Importance:** Low

Dear Mr. Rathi,

During our review of GRAS Notice No. 000991, we noted questions that need to be addressed. Please find the questions attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options.

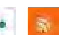
If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,
Lauren

**Lauren VieBrock, Ph.D.**
*Regulatory Review Scientist/Microbiology Reviewer*

**Center for Food Safety and Applied Nutrition**
**Office of Food Additive Safety**
**U.S. Food and Drug Administration**
Tel: 301-796-7454
lauren.viebrock@fda.hhs.gov

U.S. FOOD & DRUG
ADMINISTRATION



**GRN 991 Questions:**

1. **Please provide more information on the *Bacillus subtilis* CSSC production strain, such as deposit information and source of the strain.**

   **Notifier's response:**

   The production strain *Bacillus subtilis* CSSC used for production of chitosanase is a non-genetically modified strain. It has been a part of the culture collection of Advanced Enzyme Technologies Ltd for over two decades. The production strain has not been deposited in any culture collection center, and accordingly, a deposition number is not available for *Bacillus subtilis* CSSC.

2. **Please confirm the enzyme is secreted into the fermentation medium.**

   **Notifier's response:**

   Chitosanase produced from *Bacillus subtilis* CSSC is an extracellular enzyme and it is secreted into the fermentation medium.

3. **Please confirm that the absence of the production organism is a manufacturing specification.**

   **Notifier's response:**

   Confirmed. Advanced Enzymes routinely incorporates a series of filtration procedures to ensure the absence of the production organism in the finished enzyme preparation. It has been added to the specification for clarity. The specification has been updated as below.

| Product specification | Advanced Enzyme Technologies Ltd. | |
|---|---|---|
| | Limits | Reference Method |
| Chitosanase activity | Not less than 2,000 U/g | Internal method |
| Appearance/ Description | Light brown to brown colored powder with characteristics odor | Visual, olfactory |
| Moisture/ Loss on Drying | Not more than 10.0% | AOAC 926.08 |
| Solubility | Soluble in water | Internal method |
| Heavy metals | Not more than 30.0 ppm | AOAC 984.27 |
| Lead | Not more than 5.0 ppm | AOAC 984.27 |
| Total viable count | Not more than 10000 cfu/g | Harmonized method (IP, BP, EP and USP) |
| Total coliform | Not more than 30 cfu/g | FDA Bacteriological Analytical Manual |
| *E. coli /25g* | Negative by test | Harmonized Pharmacopoeial method (EP, BP, USP, and IP) |

| *Salmonella* spp. /25g | Negative by test | Harmonized Pharmacopoeial method (BP, USP and IP) |
|---|---|---|
| Antimicrobial activity | Absent by test | JECFA 2003 (FNP 52, Add. 11) |
| Absence of production organism | Absent by test | Internal method |

4. **Please specify the FDA BAM method used for the analysis of Total coliform.**

   **Notifier's response:**

   The method used for analysis of total coliform is adapted from FDA Bacteriological Analytical Manual Chapter 4: Enumeration of Escherichia coli and the Coliform Bacteria. The method has been attached here as Annex B.

5. **On page 17, the product specifications Table 2 lists AOAC method 984.27 for the analyses of heavy metals and lead (a method validated for the quantification of nutrient elements in infant formula using acid decomposition followed by ICP-OES analyses). Please provide results from an appropriate validated method to support the product specifications in Table 2.**

   **Notifier's response:**

   The GRAS notification only identifies one of the two methods used for quantification of heavy metals. In addition to analysis by AOAC 984.24, two of the batches of chitosanase were concurrently analyzed for heavy metals by a third-party laboratory using ICP-MS in accordance with the method set forth in AOAC 999.10 (19$^{th}$ Edition). These third-party results can be found in Annex C.

6. **On page 22, you state that "chitosanase enzymes have been shown to be effective against fungal (yeast/mold) pathogens", and then on page 23, you state that "the enzyme does not exhibit any technological effect in the final food." Please clarify these conflicting statements and provide additional information on the technical effect of WCS only vs chitosanase/WCS vs chitosanase only.**

   **Notifier's response:**

   We agree that these two statements look conflicting; however, we would like to bring to the notice that the context of these statements is different.

   *Chitosanase enzymes have been shown to be effective against fungal (yeast/mold) pathogens*

Based on the literature we have stated that the chitosanase has the ability to invade fungal pathogens as it was demonstrated by researchers earlier (Pang et al., 2021; Gao et al., 2008; Gupta et al., 2012)[1,2,3].

This statement suggests that any residual chitosanase in the final food (shrimp) may aid in the preservation of the final food (shrimp) under conditions conducive to the enzyme's function.

*The enzyme does not exhibit any technological effect in the final food.*

This statement is in context to the technological effect/action on the final food (shrimp). The shrimp shell contains chitin (8-10%, Isa et al., 2012; Islam et al., 2016)[4,5]. The water-soluble chitosan (WSC) applied to the shrimp as a preservative contains residual chitosanase that does not act on the chitin present in the shrimp shell. The lack of availability of the substrate (chitosan) in the final food (shrimp) is a reason for the residual enzyme not to perform a technological effect on the final food though it is present there.

*Additional information on the technical effect of WCS only vs chitosanase/WCS vs chitosanase only.*

Water soluble chitosan (WSC) and chitosanase both possess antimicrobial effects. Hence, it can be postulated that together they would exhibit a combined antimicrobial effect. Wang et al., 2007[6] investigated the antimicrobial effects of chitooligosaccharides (WSC) compared with chitosan and chitosanase against four species of bacteria and six species of fungi, which are mostly putrefactive microorganisms in food and aquatic preservation.

In the above-mentioned study, chitooligosaccharides, chitosan, and chitosanase all showed significantly stronger antimicrobial activities against bacteria than fungi ($p<0.001$). Inhibitory diameters of chitooligosaccharides were significantly higher than that of chitosan ($p<0.05$), but insignificantly lower than that of chitosanase ($p>0.05$).

[1] Pang, Y., Yang, J., Chen, X., Jia, Y., Li, T., Jin, J., Liu, H., Jiang, L., Hao, Y., Zhang, H., et al. An antifungal chitosanase from Bacillus subtilis SH21. Molecules. 2021, 26, 1863. https://doi.org/10.3390/molecules 26071863
[2] Gao, X-A., Ju, W-T., Jung, W-J., Park, R-D. Purification and characterization of chitosanase from Bacillus cereus D-11. Carbohydrate Polymers. 2008, 72(3), 513-520
[3] Gupta, V., Prasanna, R., Srivastava, A. K., Sharma, J. 2012. Purification and characterization of a novel antifungal endo-type chitosanase from Anabaena fertilissima. Annals of Microbiology. 62, 1089-1098
[4] Isa, M. T., Ameh, A. O., Tijjani, M., Adama, K. K. 2012. Extraction and characterization of chitin and chitosan from Nigerian shrimps. International Journal of Biological and Chemical Sciences. 6(1), 446-453.
[5] Islam, S. Z., Khan, M. and Alam, N. 2016. Production of chitin and chitosan from shrimp shell wastes. Journal of Bangladesh Agricultural University. 14(2), 253-259.
[6] Wang, Y., Zhou, P., Yu, J., Pan, X., Wang, P., Lan, W., Tao, S. 2007. Antimicrobial effect of Chitooligosaccharides Produced by Chitosanase from Pseudomonas CUY8. Asian Pacific Journal of Clinical Nutrition. 16(1), 174-177.

The results of the study showed that the antimicrobial effects of chitooligosaccharides (WSC) were similar to that of chitosanase. This study suggested that chitooligosaccharides (WSC) and chitosanase have the potential application to food and aquatic preservation.

7. **The studies in Annexes 1-4 are designated confidential. Data and information used to support a GRAS conclusion must be generally available and generally recognized and therefore cannot be confidential. Please clarify whether that information is considered confidential or if it was incorrectly designated confidential. If it is considered confidential, please provide a brief narrative to explain how experts could get to a GRAS conclusion of safety without the confidential information.**

**Notifier's response:**

Annex 1-4 contain toxicity reports carried out on chitosanase. We agree Annexes 1-4 can be treated as non-confidential.

8. **Please provide an updated Codex Alimentarius Commission reference.**

**Notifier response:**

The citation for the reference on page 23 of the GRAS notification is "Codex Alimentarius (2003). Guideline for the conduct of Food Safety assessment of foods produced using recombinant-DNA microorganisms (CAC/GL 46-2003)". (Attached as Annex D). This reference is also cited in Codex Alimentarius, 2009. Foods derived from modern biotechnology. Codex Alimentarius Commission, Joint FAO/WHO Food Standards Programme, Rome. There have been no updates in the CAC/GL 46-2003 guidelines since 2003.

9. **Please confirm that the enzyme preparation conforms to specifications established for enzyme preparations in Food Chemicals Codex.**

**Notifier's response:**

Confirmed. Chitosanase complies with the Food Chemical Codex (FCC 12). (Refer to page no. 408 for the impurities and microbial limits for the enzyme preparation)

Specifications listed in FCC 12 includes:

| **Parameters** | **Limits** |
|---|---|
| Lead | < 5 ppm |
| Coliform | < 30 CFU/g |
| *Salmonella* | Negative in 25g |

Refer to Annex E for FCC reference (Page no.5)

10. **On page 32, you state based on two cited references, "that Chitosanase can be naturally present in human diet and potentially present in the human GI tract as well." Based on our evaluation of Loncarevic et al. 2017 ("Lysozyme-Induced Degradation of Chitosan: The Characterisation of Degraded Chitosan Scaffolds") and our inability to access Somashekhar et al. 1996, it is not clear how you reached this conclusion. Furthermore, we note that your in vitro simulated gastric digestion assay and the safety studies presented in the GRN are not published, and thus would not be considered generally available and generally accepted. Finally, you state on page 23, "As the enzyme Chitosanase is not physically removed from the process, it is assumed that the enzyme used in the reaction is present in the WSC"; it is not clear whether or not the added chitosanase is inactivated during processing of the final food. Please provide a short narrative clarifying: a) residual chitosanase activity in the final food will not result in production of any reaction products that are not already part of the human diet; b) any chitosanase in the final product would be expected to undergo normal absorption and metabolism in the human body via scientifically accepted biochemical pathways.**

**Notifier's response:**

(a) <u>Residual chitosanase activity in the final food will not result in production of any reaction products that are not already part of the human diet.</u>

As an enzyme with specificity, active chitosanase works only on chitosan, producing chitooligosaccharides. Accordingly, if there is residual active chitosanase in the final food, it can only produce chitooligosaccharides that are already part of the human diet. (Rajabi, Mina. "Chitooligosaccharides in food industry." (2019): 130-131).

Moreover, we stand by our statement that the chitosanase enzyme itself can be naturally present in human diet and potentially present in the human GI tract as well. We have attached the missing article and summarize our rationale below:

> Somashekhar *et. al* 1996 (attached herein <u>Annex F</u>) describes the occurrence of chitosanase in nature. In particular, pages 35-37 state "Most bacteria and fungi secrete chitosanases extracellularly (Monaghan *et al.,* 1973; Hedges & Wolfe, 1974; Tominaga & Tsujisaka, 1975; Price & Storck, 197.5; Fenton & Eveleigh, 1981; Ohtakara *et al.,* 1984; Yabuki *et al.,* 1988; Yoshihara *et al.,* 1990; Pelletier & Sygusch, 1990; Sakai *et al,* 1991; Boucher *et al.,* 1992; Yamasaki *et al.,* 1992; Shimosaka *et al.,* 1993). Intracellular chitosanases are found in plants (Grenier *et al,* 1991; Ouakfaoui & Asselin, 1992a;b) and zygomycete fungi (Reyes *et al.,* 1985; Alfonso *et al.,* 1992). In the latter the enzyme is presumably involved in cell-wall modification. Both inducible and constitutive forms of chitosanases are known. Multiple forms of chitosanases were detected in *Bacillus megaterium* Pl, *B.licheniforrnis* UTK (Uchida *et al.,* 1992), *Mucor*

*rouxii* (Alfonso *et al.*, 1992), *Cucumis sativus* (Ouakfaoui & Asselin, 1992a) and a few plant species (Ouakfaoui & Asselin, 1992b). Osswald *et al.* (1994) have characterized 11 isoforms from *Citrus sinensis,* of which four exhibited chitinase and chitosanase activities and the remainder had chitinase activity only".

In addition, article Loncarevic *et. al* 2017 (Refer to Annex G, page no. 13) states that "In human body, chitosan degrades due to the activity of lysozyme and bacterial enzymes present in the colon".Based on the statements mentioned in these articles it can be concluded that chitosanase naturally present in bacteria, fungi and plants consumed by humans.

We note your concern about the publication of our research. Though our gastric digestion assay and safety studies referenced in the GRAS notification have not been published, we have conducted these studies in accordance with standardized regulations and regulatory guidance. Reports of our toxicity studies are annexed to the GRAS notification, and additional data can be found here as follows:

- Refer to Annex A1-A4 for toxicology studies
- Refer to Annex H for pepsin digestion study of chitosanase
- Refer to Annex I for allergenicity analysis for chitosanase from *Bacillus subtilis* CSSC by sequence homology
- Refer to Annex J for enterotoxin report of *Bacillus subtilis* CSSC strain
- Refer to Annex K for antimicrobial activity of chitosanase enzyme produced by *Bacillus subtilis* CSSC.

(b) <u>Any chitosanase in the final product would be expected to undergo normal absorption and metabolism in the human body via scientifically accepted biochemical pathways.</u>

As a protein, chitosanase is susceptible to normal human digestion of proteins, including by proteases secreted by the body. If any residual chitosanase present in the final food reached the human digestive system, it would be degraded by body proteases. Pepsin, a protease enzyme present in the human gastric system, degrades chitosanase into its component parts, as suggested by the study conducted by Advanced Enzymes and attached as Annex H. Accordingly, the component parts of the chitosanase protein (amino acids) would be expected to undergo normal absorption and metabolism in the human body.

# BAM Chapter 4: Enumeration of Escherichia coli and the Coliform Bacteria

Bacteriological Analytical Manual (BAM) Main Page (/food/laboratory-methods-food/bacteriological-analytical-manual-bam)

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**Revision History**:

- October 2020 - Section I A.3 modified to reflect that enrichment should take place at 35 ± 0.5ºC and not at 35 ± 1ºC.
- July 2017 - Chap. 4 Sec. I. E. For the completed phase of testing for *E. coli*, the incubation temperature of EC tubes has been changed from 45.5 ± 0.2°C to 44.5 ± 0.2°C. The change was made in part due to the poor ability of the control strain ATCC25922 to grown and ferment lactose to produce acid and gas at 45.5 ± 0.2°C. The use of 44.5 ± 0.2°C would also make it consistent with that used for fecal Coliform analysis in shellfish and shellfish meats (Sec. VI) as well as conditions used for *E. coli* testing by other International organizations.
- February 2013 - Shellfish analysis method revised to be consistent with the APHA Examination of seawater and shellfish, 4th ed.
- February 2013 - Membrane filter methods added to water analysis.

## Chapter Contents


- Conventional Method for Determining Coliforms and E. coli
- LST-MUG Method for Detecting E. coli in Chilled or Frozen Foods Exclusive of Bivalve Molluscan Shellfish
- Bottled Water
- Examination of Shellfish and Shellfish Meats
- Analysis for E. coli in citrus juices
- Other Methods for Enumerating Coliforms and E. coli
- References

*Escherichia coli*, originally known as *Bacterium coli* commune, was identified in 1885 by the German pediatrician, Theodor Escherich (14, 29). *E. coli* is widely distributed in the intestine of humans and warm-blooded animals and is the predominant facultative anaerobe in the bowel and part of the essential intestinal flora that maintains the physiology of the healthy host (9, 29). *E. coli* is a member of the family *Enterobacteriaceae* (15), which includes many genera, including known pathogens such as *Salmonella*, *Shigella*, and *Yersinia*. Although most strains of *E. coli* are not regarded as pathogens, they can be opportunistic pathogens that cause infections in immunocompromised hosts. There are also pathogenic strains of *E. coli* that when ingested, causes gastrointestinal illness in healthy humans (see Chap. 4A).

In 1892, Shardinger proposed the use of *E. coli* as an indicator of fecal contamination. This was based on the premise that *E. coli* is abundant in human and animal feces and not usually found in other niches. Furthermore, since *E. coli* could be easily detected by its ability to ferment glucose (later changed to lactose), it was easier to isolate than known gastrointestinal pathogens. Hence, the presence of *E. coli* in food or water became accepted as indicative of recent fecal contamination and the possible presence of frank pathogens. Although the concept of using *E. coli* as an indirect indicator of health risk was sound, it was complicated in practice, due to the presence of other enteric bacteria like *Citrobacter*, *Klebsiella* and *Enterobacter* that can also ferment lactose and are similar to *E. coli* in phenotypic characteristics, so that they are not easily distinguished. As a result, the term "coliform" was coined to describe this group of enteric bacteria. Coliform is not a taxonomic classification but rather a working definition used to describe a group of Gram-negative, facultative anaerobic rod-shaped bacteria that ferments lactose to produce acid and gas within 48 h at 35°C. In 1914, the U.S. Public Health Service adopted the enumeration of coliforms as a more convenient standard of sanitary significance.

Although coliforms were easy to detect, their association with fecal contamination was questionable because some coliforms are found naturally in environmental samples (6). This led to the introduction of the fecal coliforms as an indicator of contamination. Fecal coliform, first defined based on the works of Eijkman (12) is a subset of total coliforms that grows and ferments lactose at elevated incubation temperature, hence also referred to as thermotolerant coliforms. Fecal coliform analyses are done at 45.5°C for food testing, except for water, shellfish and shellfish harvest water analyses, which use 44.5°C (1, 3, 30). The fecal coliform group consists mostly of *E. coli* but some other enterics such as *Klebsiella* can also ferment lactose at these temperatures and therefore, be considered as fecal coliforms. The inclusion of *Klebsiella* spp in the working definition of fecal coliforms diminished the correlation of this group with fecal contamination. As a result, *E. coli* has reemerged as an indicator, partly facilitated by the introduction of newer methods that can rapidly identify *E. coli*.

Currently, all 3 groups are used as indicators but in different applications. Detection of coliforms is used as an indicator of sanitary quality of water or as a general indicator of sanitary condition in the food-processing environment. Fecal coliforms remain the standard indicator of

choice for shellfish and shellfish harvest waters; and *E. coli* is used to indicate recent fecal contamination or unsanitary processing. Almost all the methods used to detect *E. coli*, total coliforms or fecal coliforms are enumeration methods that are based on lactose fermentation (4). The Most Probable Number (MPN) method is a statistical, multi-step assay consisting of presumptive, confirmed and completed phases. In the assay, serial dilutions of a sample are inoculated into broth media. Analysts score the number of gas positive (fermentation of lactose) tubes, from which the other 2 phases of the assay are performed, and then uses the combinations of positive results to consult a statistical table (Appendix 2 (/food/laboratory-methods/bam-appendix-2-most-probable-number-serial-dilutions)), to estimate the number of organisms present. Typically only the first 2 phases are performed in coliform and fecal coliform analysis, while all 3 phases are done for *E. coli*. The 3-tube MPN test is used for testing most foods. Analysis of seawater using a multiple dilution series should not use less than 3 tubes per dilution (5 tubes are recommended); in certain instances a single dilution series using no less than 12 tubes may also be acceptable. (For additional details, see: FDA. National Shellfish Sanitation Program, Manual of Operations. 2009 Revision. DHHS/PHS/FDA, Washington DC). Likewise, analysis of bivalve molluscan shellfish should be performed using a multiple dilution MPN series whereby no fewer than 5- tubes per dilution should be used, see section IV. There is also a 10-tube MPN method that is used to test bottled water or samples that are not expected to be highly contaminated (3). Analysis of citrus juice for *E. coli* is performed as an absence/presence method, see section V.

Also, there is a solid medium plating method for coliforms that uses Violet Red Bile Agar, which contains neutral red pH indicator, so that lactose fermentation results in formation of pink colonies. There are also membrane filtration tests for coliform and fecal coliform that measure aldehyde formation due to fermentation of lactose. This chapter also includes variations of above tests that use fluorogenic substrates to detect *E. coli* (18), special tests for shellfish analysis, a brief consideration of bottled water testing and a method for testing large volumes of citrus juices for presence of *E. coli* in conjunction with the Juice HACCP rule.

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## I. Conventional Method for coliforms, fecal coliforms and *E. coli*

A. **Equipment and materials**

1. Covered water bath, with circulating system to maintain temperature of 44.5 ± 0.2°C. The temperature for water baths for the shellfish program is 44.5°C ± 0.2°C. Water level should be above the medium in immersed tubes.
2. Immersion-type thermometer, 1-55°C, about 55 cm long, with 0.1°C subdivisions, certified by National Institute of Standards and Technology (NIST), or equivalent Incubator, 35 ± 0.5°C.
3. Balance with capacity of ≥2 kg and sensitivity of 0.1 g

4. Blender and blender jar (**see** Chapter 1)
5. Sterile graduated pipets, 1.0 and 10.0 mL
6. Sterile utensils for sample handling (**see** Chapter 1)
7. Dilution bottles made of borosilicate glass, with polyethylene screw caps equipped with Teflon liners. Commercially prepared dilution bottles containing sterile Butterfield's phosphate buffer can also be used.
8. Quebec colony counter, or equivalent, with magnifying lens
9. Longwave UV light [~365 nm], not to exceed 6 W.
10. pH meter

B. **Media (/food/laboratory-methods/media-index-bam) and Reagents (/food/laboratory-methods/reagents-index-bam)**

1. Brilliant green lactose bile (BGLB) broth, 2% (M25 (/food/laboratory-methods/bam-media-m25-brilliant-green-lactose-bile-broth))
2. Lauryl tryptose (LST) broth (M76 (/food/laboratory-methods/bam-media-m76-lauryl-tryptose-lst-broth))
3. Lactose Broth (M74 (/food/laboratory-methods/bam-media-m74-lactose-broth))
4. EC broth (M49 (/food/laboratory-methods/bam-media-m49-ec-broth))
5. Levine's eosin-methylene blue (L-EMB) agar (M80 (/food/laboratory-methods/bam-media-m80-levines-eosin-methylene-blue-l-emb-agar))
6. Tryptone (tryptophane) broth (M164 (/food/laboratory-methods/bam-media-m164-tryptone-tryptophane-broth-1))
7. MR-VP broth (M104 (/food/laboratory-methods/bam-media-m104-mr-vp-broth))
8. Koser's citrate broth (M72 (/food/laboratory-methods/bam-media-m72-kosers-citrate-broth))
9. Plate count agar (PCA) (standard methods) (M124 (/food/laboratory-methods/bam-media-m124-plate-count-agar-standard-methods))
10. Butterfield's phosphate-buffered water (R11 (/food/laboratory-methods/bam-r11-butterfields-phosphate-buffered-dilution-water)) or equivalent diluent

    **(Note**: This same formulation is referred to as Buffered Dilution Water in American Public Health Association. 1970. Recommended Procedures for the Examination of Seawater and Shellfish, 4th ed. APHA, Washington, DC., p14-15)
11. Kovacs' reagent (R38 (/food/laboratory-methods/bam-r38-kovacs-reagent))
12. Voges-Proskauer (VP) reagents (R89 (/food/laboratory-methods/bam-r89-voges-proskauer-vp-test-reagents))

13. Gram stain reagents (R32 (/food/laboratory-methods/bam-r32-gram-stain))
14. Methyl red indicator (R44 (/food/laboratory-methods/bam-r44-methyl-red-indicator))
15. Violet red bile agar (VRBA) (M174 (/food/laboratory-methods/bam-media-m174-violet-red-bile-agar-vrba))
16. VRBA-MUG agar (M175 (/food/laboratory-methods/bam-media-m175-violet-red-bile-mug-agar))
17. EC-MUG medium (M50 (/food/laboratory-methods/bam-media-m50-ec-mug-medium))
18. Lauryl tryptose MUG (LST-MUG) broth (M77 (/food/laboratory-methods/bam-media-m77-lauryl-tryptose-mug-lst-mug-broth))
19. Peptone Diluent, 0.5% (R97 (/food/laboratory-methods/bam-r97-peptone-diluent-05))

C. **MPN - Presumptive test for coliforms, fecal coliforms and *E. coli***

Weigh 50 g of food into sterile high-speed blender jar (see Chapter 1 and current FDA compliance programs for instructions on sample size and compositing) Frozen samples can be softened by storing for <18 h at 2-5°c, but do not thaw. Add 450 mL of Butterfield's phosphate-buffered water and blend for 2 min. If <50 g of sample are available, weigh portion that is equivalent to half of the sample and add sufficient volume of sterile diluent to make a 1:10 dilution. The total volume in the blender jar should completely cover the blades.

Prepare decimal dilutions with sterile Butterfield's phosphate diluent or equivalent. Number of dilutions to be prepared depends on anticipated coliform density. Shake all suspensions 25 times in 30 cm arc or vortex mix for 7 s. Using at least 3 consecutive dilutions, inoculate 1 mL aliquots from each dilution into 3 LST tubes for a 3 tube MPN analysis (other analysis may require the use of 5 tubes for each dilution; See IV). Lactose Broth may also be used. For better accuracy, use a 1 mL or 5 mL pipet for inoculation. Do not use pipets to deliver<10% of their total volume; eg. a 10 mL pipet to deliver 0.5 mL. Hold pipet at angle so that its lower edge rests against the tube. Not more than 15 min should elapse from time the sample is blended until all dilutions are inoculated in appropriate media.

Incubate LST tubes at 35°C± 0.5°C . Examine tubes and record reactions at 24 ± 2 h for gas, i.e., displacement of medium in fermentation vial or effervescence when tubes are gently agitated. Re-incubate gas-negative tubes for an additional 24 h and examine and record reactions again at 48 ± 3 h. Perform confirmed test on all presumptive positive (gas) tubes.

D. **MPN - Confirmed test for coliforms**

From each gassing LST or lactose broth tube, transfer a loopful of suspension to a tube of BGLB broth, avoiding pellicle if present. (a sterile wooden applicator stick may also be used for these transfers). Incubate BGLB tubes at 35°C ± 0.5°C and examine for gas production at 48 ± 3 h. Calculate most probable number (MPN) (see Appendix 2 (/food/laboratory-methods/bam-appendix-2-most-probable-number-serial-dilutions)) of coliforms based on proportion of confirmed gassing LST tubes for 3 consecutive dilutions.

E. **MPN - Confirmed test for fecal coliforms and *E. coli***

From each gassing LST or Lactose broth tube from the Presumptive test, transfer a loopful of each suspension to a tube of EC broth (a sterile wooden applicator stick may also be used for these transfers). Incubate EC tubes 24 ± 2 h at 44.5°C and examine for gas production. If negative, reincubate and examine again at 48 ± 2 h. Use results of this test to calculate fecal coliform MPN. To continue with *E. coli* analysis, proceed to Section F below. The EC broth MPN method may be used for seawater and shellfish since it conforms to recommended procedures (1).

F. **MPN - Completed test for *E. coli*.**

To perform the completed test for *E. coli*, gently agitate each gassing EC tube, remove a loopful of broth and streak for isolation on a L-EMB agar plate and incubate for 18-24 h at 35°C ± 0.5°C . Examine plates for suspicious *E. coli* colonies, i.e., dark centered and flat, with or without metallic sheen. Transfer up to 5 suspicious colonies from each L-EMB plate to PCA slants, incubate them for 18-24 h at 35°C ± 0.5°C and use for further testing.

**NOTE:** Identification of any 1 of the 5 colonies as *E. coli* is sufficient to regard that EC tube as positive; hence, not all 5 isolates may need to be tested.

Perform Gram stain. All cultures appearing as Gram-negative, short rods should be tested for the IMViC reactions below and also re-inoculated back into LST to confirm gas production.

Indole production. Inoculate tube of tryptone broth and incubate 24 ± 2 h at 35°C ± 0.5°C . Test for indole by adding 0.2-0.3 mL of Kovacs' reagent. Appearance of distinct red color in upper layer is positive test.

Voges-Proskauer (VP)-reactive compounds. Inoculate tube of MR-VP broth and incubate 48 ± 2 h at 35°C± 0.5°C . Transfer 1 mL to 13 × 100 mm tube. Add 0.6 mL α-naphthol solution and 0.2 mL 40% KOH, and shake. Add a few crystals of creatine. Shake and let stand 2 h. Test is positive if eosin pink color develops.

Methyl red-reactive compounds. After VP test, incubate MR-VP tube additional 48 ± 2 h at 35°C± 0.5°C . Add 5 drops of methyl red solution to each tube. Distinct red color is positive test. Yellow is negative reaction.

Citrate. Lightly inoculate tube of Koser's citrate broth; avoid detectable turbidity. Incubate for 96 h at 35°C ± 0.5°C . Development of distinct turbidity is positive reaction.

Gas from lactose. Inoculate a tube of LST and incubate 48 ± 2 h at 35°C ± 0.5°C . Gas production (displacement of medium from inner vial) or effervescence after gentle agitation is positive reaction.

**Interpretation:** All cultures that (a) ferment lactose with gas production within 48 h at 35°C, (b) appear as Gram-negative nonsporeforming rods and (c) give IMViC patterns of ++-- (biotype 1) or -+-- (biotype 2) are considered to be *E. coli*. Calculate MPN (see Appendix 2) of *E. coli* based on proportion of EC tubes in 3 successive dilutions that contain *E. coli*.

**NOTE:** Alternatively, instead of performing the IMViC test, use API20E or the automated VITEK biochemical assay to identify the organism as *E. coli*. Use growth from the PCA slants and perform these assays as described by the manufacturer.

G. **Solid medium method - Coliforms**

Prepare violet red bile agar (VRBA) according to manufacturer's instructions. Cool to 48°C before use. Prepare, homogenize, and decimally dilute sample as described in section I. C above so that isolated colonies will be obtained when plated. Transfer two 1 mL aliquots of each dilution to petri dishes, and use either of the following two pour plating methods, depending on whether injured or stressed cells are suspected to be present (1).

Pour 10 mL VRBA tempered to 48°C into plates, swirl plates to mix, and let solidify. To prevent surface growth and spreading of colonies, overlay with 5 mL VRBA, and let solidify. If resuscitation is necessary, pour a basal layer of 8-10 mL of tryptic soy agar tempered to 48°C. Swirl plates to mix, and incubate at room temperature for 2 ± 0.5 h. Then overlay with 8-10 mL of melted, cooled VRBA and let solidify.

Invert solidified plates and incubate 18-24 h at 35°C. Incubate dairy products at 32°C (2). Examine plates under magnifying lens and with illumination. Count purple-red colonies that are 0.5 mm or larger in diameter and surrounded by zone of precipitated bile acids. Plates should have 25-250 colonies. To confirm that the colonies are coliforms, pick at least 10 representative colonies and transfer each to a tube of BGLB broth. Incubate tubes at 35°C. Examine at 24 and 48 h for gas production.

**NOTE:** If gas-positive BGLB tube shows a pellicle, perform Gram stain to ensure that gas production was not due to Gram-positive, lactose-fermenting bacilli.

Determine the number of coliforms per gram by multiplying the number of suspect colonies by percent confirmed in BGLB by dilution factor.

Alternatively, *E. coli* colonies can be distinguished among the coliform colonies on VRBA by adding 100 µg of 4-methyl-umbelliferyl-β-D-glucuronide (MUG) per mL in the VRBA overlay. After incubation, observe for bluish fluorescence around colonies under longwave UV light. (see LST-MUG section II for theory and applicability.)

H. **Membrane Filtration (MF) Method - coliforms: see Section III. Bottled Water.**

Food homogenates will easily clog filters, hence MF are most suitable for analysis of water samples; however, MF may be used in the analysis of liquid foods that do not contain high levels of particulate matter such as bottled water (see Section III for application of MF).

## II. LST-MUG Method for Detecting *E. coli* in Chilled or Frozen Foods Exclusive of Bivalve Molluscan Shellfish

The LST-MUG assay is based on the enzymatic activity of β-glucuronidase (GUD), which cleaves the substrate 4-methylumbelliferyl β-D-glucuronide (MUG), to release 4-methylumbelliferone (MU). When exposed to longwave (365 nm) UV light, MU exhibits a bluish fluorescence that is easily visualized in the medium or around the colonies. Over 95% of *E. coli* produces GUD, including anaerogenic (non-gas-producing) strains. One exception is enterohemorrhagic *E. coli* (EHEC) of serotype O157:H7, which is consistently GUD negative (11, 17). The lack of GUD phenotype in O157:H7 is often used to differentiate this serotype from other *E. coli*, although GUD positive variants of O157:H7 do exist (24, 26). The production of GUD by other members of the family *Enterobacteriaceae* is rare, except for some shigellae (44 -58%) and salmonellae (20-29%) (18, 27). However, the inadvertent detection of these pathogens by GUD-based assays is not considered a drawback from a public health perspective. Expression of GUD activity is affected by catabolite repression (8) so on occasion, some *E. coli* are GUD-negative, even though they carry the *uid*A gene (*gus*A) that encodes for the enzyme (19). In most analyses however, about 96% of *E. coli* isolates tested are GUD-positive without the need for enzyme induction (27).

MUG can be incorporated into almost any medium for use in detecting *E. coli*. But some media such as EMB, which contain fluorescent components, are not suitable, as they will mask the fluorescence of MU. When MUG is incorporated into LST medium, coliforms can be enumerated on the basis of gas production from lactose and *E. coli* are presumptively identified by fluorescence in the medium under longwave UV light, thus it is capable of providing a presumptive identification of *E. coli* within 24 h (18, 28). The LST-MUG method described below has been adopted as Official Final Action by the AOAC for testing for *E. coli* in chilled or frozen foods, exclusive of shellfish (28). See Sec. IV.4. D. for precautions in using MUG in testing shellfish. For information on MUG assay contact, Dr. Bill Burkhardt III (email William.Burkhardt@fda.hhs.gov (mailto:William.Burkhardt@fda.hhs.gov) ), FDA, CFSAN, Dauphin Island, AL, 36528; 251-406-8125

**CAUTION:** To observe for fluorescence, examine inoculated LST-MUG tubes under longwave (365 nm) UV light in the dark. A 6-watt hand-held UV lamp is adequate and safe. When using a more powerful UV source, such as a 15-watt fluorescent lamp, wear protective glasses or goggles. Also, prior to use in MUG assays, examine all glass tubes for auto fluorescence. Cerium oxide, which is sometimes added to glass as a quality control measure, will fluoresce under UV light and interfere with the MUG test (25). The use of positive and negative control strains for MUG reaction is essential.

A. **Equipment and material: see section I.A above and in addition**,

1. New, disposable borosilicate glass tubes (100 × 16 mm)
2. New, disposable borosilicate glass Durham vials (50 × 9 mm) for gas collection
3. Longwave UV lamp, not to exceed 6-watt

B. **Media and reagents: see section I.B above**

C. **Presumptive LST-MUG test for *E. coli.***

Prepare food samples and perform the MPN Presumptive test as described in section I.C. above, except use LST-MUG tubes instead of LST. Be sure to inoculate one tube of LST-MUG with a known GUD-positive *E. coli* isolate as positive control (ATCC 25922). In addition, inoculate another tube with a culture of *Enterobacter aerogenes* (ATCC 13048) culture of Enterobacter aerogenes (ATCC 13048) or a *Klebsiella pneumoniae* strain as negative control, to facilitate differentiation of sample tubes that show only growth from those showing both growth and fluorescence. Incubate tubes for 24 to 48 ± 2 h at 35°C. Examine each tube for growth (turbidity, gas) then examine tubes in the dark under longwave UV lamp (365 nm). A bluish fluorescence is a positive presumptive test for *E. coli*. Studies by Moberg et al. (28) show that a 24 h fluorescence reading is an accurate predictor of *E. coli* and can identify 83-95% of the *E. coli*-positive tubes. After 48 h of incubation, 96-100% of *E. coli*-positive tubes can be identified (28). Perform a confirmed test on all presumptive positive tubes by streaking a loopful of suspension from each fluorescing tube to L-EMB agar and incubate 24 ± 2 h at 35°C. Follow protocols outlined in I. F, above, for Completed test for *E. coli*. Calculate MPN of *E. coli* based on combination of confirmed fluorescing tubes in 3 successive dilutions.

## III. Examination of Bottled Water

Consumption of bottled water is increasing rapidly worldwide. In the U.S. alone, over 3.6 billion gallons of bottled water were consumed in 1998 (International Bottled Water Association, Alexandria, VA). Unlike potable water, which is regulated by the U.S. EPA, bottled water is legally classified as food in the U.S. and regulated by the FDA (Federal Register. 1995. 21 CFR Part 103 et al. beverages: bottled water; final rule. 60(218) 57076-57130). FDA defines bottled water as "water that is intended for human consumption and that is sealed in bottles or other containers with no added ingredients except that it may contain safe and suitable antimicrobial agents" and, within limitations, some added fluoride. Bottled water may be used as a beverage by itself or as an ingredient in other beverages. These regulations do not apply to soft drinks or similar beverages. In addition to "bottled water" or "drinking water", in 21 CFR Part 103 FDA also defines various types of bottled water that meet certain criteria. These identities include "artesian or artesian well water", "ground water", mineral water", "purified or demineralized water", "sparkling bottled water", "spring water" and "well water". Additionally "sterile water" is defined as water that meets the requirements under the "Sterility Test" in the United States Pharmacopeia.

Coliform organisms are not necessarily pathogens and are rarely found in bottled water, however, they serve as an indicator of insanitation or possible contamination. Surveys have shown that coliforms are useful indicators of bottled water quality, but some countries also monitor additional microbial populations as indicators of bottle water quality (10, 33). Under the current bottled water quality standard, FDA has established a microbiological quality requirement that is based on coliform detection levels. These levels may be obtained by membrane filtration (MF) or by 10-tube MPN analysis of ten 10-mL analytical units. For information on bottled water methods contact Dr. Bill Burkhardt III (email William.Burkhardt@fda.hhs.gov (mailto:William.Burkhardt@fda.hhs.gov) ), FDA, CFSAN, Dauphin Island, AL, 36528; 251-406-8125

A. **Equipment and Materials.**

1. Incubator at 35° ± 0.5°C.
2. Membrane filtration units (filter base and funnels): glass, plastic, or stainless steel; wrapped in foil or paper and sterilized.
3. Ultraviolet sterilization chamber for sterilizing filter base and funnels (optional).
4. Filter manifold or vacuum flask to hold filter funnels.
5. Vacuum source (line vacuum, electric vacuum pump or water aspirator).
6. Membrane filters; sterile, white, gridded, 47 mm diameter, 0.45 µm pore size (or equivalent, as specified by the manufacturer) for enumeration of bacteria.
7. Petri dishes, sterile, plastic, 50 × 12 mm, with tight fitting lids.
8. Forceps designed to transfer membranes without damage.

B. **Culture media.**

1. Lauryl sulfate tryptose (LST) broth (M-76 (/food/laboratory-methods/bam-media-m76-lauryl-tryptose-lst-broth)).
2. Brilliant green lactose bile broth (BGLB) (M-25 (/food/laboratory-methods/bam-media-m25-brilliant-green-lactose-bile-broth)).
3. M-Endo Medium (BD#274930) (M-196 (/food/laboratory-methods/bam-media-m-196-mendo-mf-medium-bd-274930)).
4. LES-Endo Agar (BD#273620) (M-197 (/food/laboratory-methods/bam-media-m-197-les-endo-agar-bd-273620)).

C. **Ten tube MPN coliform test - Presumptive and Confirmed procedures.**

For routine examination of bottled water, take 100 mL of sample and inoculate 10 tubes of 2X LST (10 mL of medium) with 10 mL of undiluted sample each. Incubate tubes at 35°C. Examine tubes at 24 ± 2 h for growth and gas formation as evidenced by displacement of medium in fermentation vial or effervescence when tubes are gently agitated. If negative at

24 h, reincubate tubes for an additional 24 h and examine again for gas. Perform a confirmed test on all presumptive positive (gassing) tubes as follows: gently agitate each positive LST tube and, using a 3.0 - 3.5 mm sterile loop, transfer one or more loopfuls of suspension to a tube of BGLB broth. Sterile wooden applicator sticks may also be used for transfer by inserting it at least 2.5 cm into the broth culture. Incubate BGLB tubes for 48 ± 2 h at 35°C. Examine for gas production and record. Calculate MPN using 10 tube MPN Table (9221.III), p. 9-52, Standard Methods for the Examination of Water and Wastewater (3).

**NOTE**: if a sample is found to contain coliforms (at any level) follow procedure outlined in Sec. I. F. above to determine if it is *E. coli*. Bottled water is not permitted to contain *E. coli*.

D. **Membrane filter method for coliforms.**

Filter 100 mL of test sample and transfer the filter to M-Endo medium (M-196 (/food/laboratory-methods/bam-media-m-196-mendo-mf-medium-bd-274930)) or LES Endo Agar (M-197 (/food/laboratory-methods/bam-media-m-197-les-endo-agar-bd-273620)) and incubate at 35 °C± 0.5°C for 22-24 h. Count colonies that are pink to dark red with a green metallic surface sheen. The sheen may vary from pinpoint to complete coverage of the colony. Use of a low power, dissecting-type microscope to examine filters is recommended.

Confirmation - If there are 5 to 10 sheen colonies on the filter, confirm all by inoculating growth from each sheen colony into tubes of LST and incubate at 35 °C± 0.5°C for 48 h. If the number of sheen colonies exceeds 10, randomly select and confirm 10 colonies that are representative of all sheen colonies. Any gas positive LST tubes should be sub cultured to BGLB and incubated at 35°C± 0.5°C for 48 hr. Gas production in BGLB within 48 h is a confirmed coliform test. Report results as number of coliform colonies per 100 mL. NOTE: Standard Method, 1998, 20th ed, p. 9-60 (3), allows for simultaneous inoculation of LST and BGLB during verification. However, BGLB is somewhat inhibitory so the method described above, where samples are sub cultured from LST into BGLB is regarded as a more sensitive verification assay and therefore, recommended.

**NOTE**: if a sample is found to contain coliforms (at any level) follow procedure outlined in Sec. I. F. above to determine if it is *E. coli*. Bottled water is not permitted to contain *E. coli*.

## IV. Examination of Shellfish and Shellfish Meats

The official FDA procedure for bacteriological analysis of domestic and imported bivalve molluscan shellfish is fully and properly described in the APHA's Recommended Procedures for the Examination of Sea Water and Shellfish, 4th ed. 1970 (1). The methods, including the conventional 5-tube MPN for coliform, fecal coliform and standard total plate count for bacteria (see Part III, APHA's Recommended Procedures the Examination of Sea Water and Shellfish, 4th ed. 1970 (1), are described below for examining shell stock, fresh-shucked meats, fresh-shucked frozen shellfish, and shellfish frozen on the half shell. These procedures do not apply to

the examination of crustaceans (crabs, lobsters, and shrimp) or to processed shellfish meats such as breaded, shucked, pre-cooked, and heat-processed products (see section I. C. this chapter). Also, there are many methods that are used for testing for shellfish harvest and environmental water for fecal coliforms. One example, the mTEC agar (M-198 (/food/laboratory-methods/bam-media-m198-mtec-agar-bd-233410)) is a suitable membrane filter medium for enumerating fecal coliforms in marine and estuarine waters. Briefly, following the filtration of 100 ml of water, the filter funnels should be rinsed twice with approx. 20 ml of PBS. The filter is then transferred onto mTEC agar and incubated for 22-24 h at 44.5°C in Ethyfoam. All yellow, yellow-green or yellow-brown colonies are counted as fecal coliforms. Only plates having fewer than 80 colonies are counted. However, analysis of environmental waters will not be covered in detail here, as environmental water analyses are done by the U.S. EPA (3) and the quality of shellfish harvest waters are mainly the responsibilities of each State's Shellfish Control Authorities (20).

A. **Sample Preparation**

Using 10-12 shellfish, obtain 200 g of shellfish liquor and meat. Blend 2 min, with 200 mL sterile phosphate buffered dilution water or 0.5% peptone water (R97 (/food/laboratory-methods/bam-r97-peptone-diluent-05)) to yield a 1:2 dilution of sample. Analysis of the ground sample must begin within 2 min after blending. Make serial dilutions in 0.5% sterile peptone water or sterile phosphate buffered dilution water.

B. **MPN - Presumptive and Confirmed Test for Coliform**

Use Lactose Broth (M74 (/food/laboratory-methods/bam-media-m74-lactose-broth)) or Lauryl Tryptose Broth (M76 (/food/laboratory-methods/bam-media-m76-lauryl-tryptose-lst-broth)), at single strength in 10 ml volumes. For 5-tube MPN analysis, inoculate the 5 tubes at each dilution as follows:

To each of 5 tubes, add 2 mL of the blended homogenate (equivalent to 1 g of shellfish).

To each of 5 tubes, add 1 mL of 1:10 dilution of homogenate (0.1 g shellfish).

To each of 5 tubes, add 1 mL of 1:100 dilution of homogenate (0.01 g shellfish).

To each of 5 tubes, add 1 mL of 1:1000 dilution of homogenate (0.001 g shellfish).

Further dilutions may be necessary to avoid indeterminate results. Incubate tubes at 35°C ± 0.5°C then follow instructions in section 1.C and perform Confirmed test as in 1.D above, under "Conventional Method for Coliforms, fecal coliforms and *E. coli*". Calculate MPN as described in section 1.D above, except that shellfish analysis specifies that the coliform density be expressed as MPN per 100 g of sample rather than per g.

C. **MPN - Presumptive and Confirmed Test for Fecal Coliforms in Shellfish**

Perform presumptive test as described in section II above. To confirm positive tubes, transfer one loopful from gas positive LST tubes to EC broth and incubate in a covered circulating waterbath at 44.5°±0.2°C for 24 ± 2 hr. Gas production in EC is a positive confirmed test for fecal coliforms. Calculate the MPN per 100 g for fecal coliforms as described above for coliform.

D. **MPN - EC-MUG Method for Determining *E. coli* in Shellfish Meats**

The MUG assay for β-glucuronidase (GUD) described above for detecting *E. coli* in chilled and frozen food can also be used for testing for *E. coli* in shellfish meats; but with slight modifications. This is due to the fact that foods such as shellfish meats contain natural GUD activity (32). As a result, oyster homogenate inoculated directly into LST-MUG tubes in the Presumptive phase of the MPN test can cause false positive fluorescence reactions. Hence, in the analysis of *E. coli* in shellfish meats, the MUG reagent is added to the EC medium and used in the confirmatory phase of the assay. The EC-MUG tubes, incubated at 44.5°C ± 0.2°C, can be used in the confirmatory phase of a conventional 5-tube MPN assay to determine fecal coliform levels in shellfish meats, then by examining tubes for fluorescence under longwave UV, an *E. coli* MPN can also be readily obtained (32).

See section 1.A and 1.B above for materials and reagents required. Use commercially prepared dehydrated EC-MUG, or prepare medium by adding MUG to EC broth (0.05 g/L) (M50 (/food/laboratory-methods/bam-media-m50-ec-mug-medium)). Several sources of MUG compound are suitable: Marcor Development Corp., Carlstadt, NJ; Biosynth International, Itasca, IL; Sigma Chemical Co., St. Louis, MO and Hach Chemical, Loveland, CO. Dispense 5 mL into new disposable borosilicate glass tubes (100 × 16 mm) containing, new disposable borosilicate glass Durham vials (50 × 9 mm) for gas collection. Sterilize EC-MUG broth tubes at 121°C for 15 min; store up to 1 week at room temperature or up to a month under refrigeration.

Perform the 5-tube MPN Presumptive and Confirmed Test for Fecal Coliforms in Shellfish as described above in Section 3, except use EC-MUG tubes instead of EC for the confirmed test. Determination of fluorescence in EC-MUG broth requires the use of 3 control tubes, one inoculated with *E. coli* as positive control; one with *Enterobacter aerogenes* (ATCC 13048) or *K. pneumoniae* as negative control; and an uninoculated tube as EC-MUG medium batch control. Inoculate the positive and negative controls at the time when Confirmed test is being performed and incubate all tubes at 44.5°C ± 0.2°C for 24 h.

Read fluorescence as described above under LST-MUG assay. Note that some (<10%)>*E. coli* are anaerogenic (gas-negative), but should be MUG-positive. Include all fluorescence positive tubes in the *E. coli* MPN calculations. Determine *E. coli* MPN/100g from the tables in the BAM (Appendix 2) using combination of fluorescence positive tubes at each dilution.

**NOTE:** If analysis is to determine compliance with established *E. coli* limits, it will be necessary to confirm the presence of *E. coli* in MUG positive tubes.

## V. Analysis for *E. coli* in citrus juices

Analysis for *E. coli* has been implemented to identify potentially contaminated juices or for verifying the effectiveness of HACCP during processing of unpasteurized juices (21 CFR Part 120, Vol. 66, No. 13, January 19, 2001). The standard method commonly used for testing for *E. coli* is the MPN however, it does not seem adequate for juice testing because of the acidity (pH 3.6 to 4.3) of juices, which can interfere with the test, plus it only allows for testing 3.33 mL of sample. Unlike most *E. coli* methods, which are enumeration assays, the following method is a simple Presence/Absence test that can examine 10-mL volume of juices (34, 35). This assay, designated as modified ColiComplete (CC) Method, is a modification of AOAC Official Method 992.30, which uses MUG for detection of *E. coli* (see Section on LST-MUG Method for details).

A. **Equipment and materials**

1. Covered water bath, with circulating system to maintain temperature of 44.5 ± 0.2°C.
   Water level should be above the medium in immersed tubes.
2. Incubator, 35 ± 0.5°C
3. Longwave UV light [~365 nm], not to exceed 6 W.

B. **Media and reagents:**

1. Universal Preenrichment Broth (UPEB) (M188 (/food/laboratory-methods/bam-media-m188-universal-preenrichment-broth)) or can be purchased from BD(#223510)
2. EC medium (M49 (/food/laboratory-methods/bam-media-m49-ec-broth))
3. ColiComplete (CC) discs (#10800) - BioControl, Bellevue, WA

C. **Sample preparation, enrichment and analysis**

Perform assay in duplicate. Aseptically, inoculate 10-mL portion of juice into 90 mL of UPEB and incubate at 35°C ± 0.5°C for 24 h. After enrichment, mix and transfer 1-mL from each UPEB enrichment broth into 9 mL of EC broth containing a CC disc. Incubate EC/CC broth tubes at 44.5± 0.2°C in a circulating water bath for 24 ± 2 h. Include a tube inoculated with a MUG (+) *E. coli* strain as positive control and another with *K. pneumoniae* or *Enterobacter aerogenes* (ATCC 13048) as negative control. Examine tubes in the dark and under long wave UV light. The presence of blue fluorescence in either tube is indicative that *E. coli* is present in the sample. Note: The CC discs also contain X-gal, which when cleaved by β-galactosidase will yield blue color on or around the disc. This reaction is analogous to measuring acid/gas production from fermentation of lactose hence, the presence of blue color is indicative of coliforms.

## VI. Other Methods for Enumerating Coliforms and *E. coli*

There are many other methods for enumerating coliforms and *E. coli* , including several that uses fluorogenic reagents like MUG or other chromogenic substrates for presumptive detection and identification of coliform and *E. coli* in foods. Many of these tests, such as the Petrifilm dry rehydratable film, the hydrophobic grid membrane filter/MUG (HGMF/MUG) method (13), ColiComplete disc (16), Colilert (AOAC 991.15), have been evaluated by collaborative studies and adopted as official first or final action by the AOAC. There are also many modifications of the membrane filtration assays that have been developed for testing for coliform, fecal coliform and *E. coli* and some of these may be useful in testing foods such as milk and beverages, but they are used mostly for water, environmental waters, and shellfish harvest waters analysis (5, 7, 20, 22, 23, 31).

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Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998. Chapter 4.

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CIN : U74140MH1989PTC052930

# Test Report

**Report No : TUV(I)/4272/14-15/0101400634**

**Date : 17 Oct 2014**

Name & Address of Customer : Advanced Enzymes Technologies Ltd.
Plot No A 61/62, Malegaon MIDC, Sinnar,
Nashik

Reg No. : 4272/14-15

CA No. : 0101400634

Date of sample receipt : 13 Oct 2014

Date(s) of analysis : 13 Oct 2014 -16 Oct 2014

Sample Drawn by : Customer

| Sl No | Test Name | Result | Unit | LOQ/LOD | Test Method |
|---|---|---|---|---|---|
| | **Sample Name : Chitosanase**<br>Batch No :061434 | | MFD : June 14 | | **CA No : 0101400634** |
| | **Heavy Metals** | | | | |
| 1 | Arsenic | <LOQ | mg/kg | <0.1 | Based on AOAC 984.27 & 999.10 19th Edition By ICP-MS |
| 2 | Cadmium | <LOQ | mg/kg | <0.1 | Based on AOAC 984.27 & 999.10 19th Edition By ICP-MS |
| 3 | Lead | <LOQ | mg/kg | <0.1 | Based on AOAC 984.27 & 999.10 19th Edition By ICP-MS |
| 4 | Mercury | <LOQ | mg/kg | <0.025 | Based on AOAC 984.27 & 999.10 19th Edition By ICP-MS |

**LOQ-Limit of Quantification**
**LOD-Limit of Detection**

Verified by

Bharat Ugare
Incharge Instrumentation (GC)

Authorized by

Lalitkumar Thakur
Section Incharge – Instrumentation

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# Test Report

**Report No : TUV(I)/4272/14-15/0101400635**

**Date : 17 Oct 2014**

| | | |
|---|---|---|
| Name & Address of Customer | : | Advanced Enzymes Technologies Ltd.<br>Plot No A 61/62, Malegaon MIDC, Sinnar,<br>Nashik |
| Reg No. | : | 4272/14-15 |
| CA No. | : | 0101400635 |
| Date of sample receipt | : | 13 Oct 2014 |
| Date(s) of analysis | : | 13 Oct 2014 -16 Oct 2014 |
| Sample Drawn by | : | Customer |

| Sl No | Test Name | Result | Unit | LOQ/LOD | Test Method |
|---|---|---|---|---|---|
| | **Sample Name : Chitosanase**<br>Batch No : 101423 | | MFD : Oct 14 | | **CA No : 0101400635** |
| | **Heavy Metals** | | | | |
| 1 | Arsenic | <LOQ | mg/kg | <0.1 | Based on AOAC 984.27 & 999.10 19th Edition By ICP-MS |
| 2 | Cadmium | <LOQ | mg/kg | <0.1 | Based on AOAC 984.27 & 999.10 19th Edition By ICP-MS |
| 3 | Lead | <LOQ | mg/kg | <0.1 | Based on AOAC 984.27 & 999.10 19th Edition By ICP-MS |
| 4 | Mercury | <LOQ | mg/kg | <0.025 | Based on AOAC 984.27 & 999.10 19th Edition By ICP-MS |

**LOQ-Limit of Quantification**
**LOD-Limit of Detection**

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# GUIDELINE FOR THE CONDUCT OF FOOD SAFETY ASSESSMENT OF FOODS PRODUCED USING RECOMBINANT-DNA MICROORGANISMS

***CAC/GL 46-2003***

## SECTION 1 – SCOPE

1. This Guideline supports the Principles for the Risk Analysis of Foods Derived from Modern Biotechnology and addresses safety and nutritional aspects of foods produced through the actions of recombinant-DNA microorganisms.[1] The recombinant-DNA microorganisms that are used to produce these foods are typically derived using the techniques of modern biotechnology from strains that have a history of safe, purposeful use in food production. However, in instances where the recipient strains do not have a history of safe use their safety will have to be established.[2] Such food and food ingredients may contain viable or non-viable recombinant-DNA microorganisms or may be produced by fermentation using recombinant-DNA microorganisms from which the recombinant-DNA microorganisms may have been removed.

2. Recognizing that the following issues may have to be addressed by other bodies or other instruments, this document does not address:

- safety of microorganisms used in agriculture (for plant protection, biofertilizers, in animal feed or food derived from animals fed the feed etc.);
- risks related to environmental releases of recombinant-DNA microorganisms used in food production;
- safety of substances produced by microorganisms that are used as additives or processing aids, including enzymes for use in food production[3];
- specific purported health benefits or probiotic effects that may be attributed to the use of microorganisms in food; or
- issues relating to the safety of food production workers handling recombinant-DNA microorganisms.

3. A variety of microorganisms used in food production have a long history of safe use that predates scientific assessment. Few microorganisms have been assessed scientifically in a manner that would fully characterize all potential risks associated with the food they are used to produce, including, in some instances, the consumption of viable microorganisms. Furthermore, the Codex principles of risk analysis, particularly those for risk assessment, are primarily intended to apply to discrete chemical entities such as food additives and pesticide residues, or specific chemical or microbial contaminants that have identifiable hazards and risks; they were not originally intended to apply to intentional uses of microorganisms in food processing or in the foods transformed by microbial fermentations. The safety assessments that have been conducted have focused primarily on the absence of properties associated with pathogenicity in these microorganisms and the absence of reports of adverse events attributed to ingestion of these microorganisms, rather than evaluating the results of prescribed studies. Further, many foods contain substances that would be considered harmful if subjected to conventional approaches to safety testing. Thus, a more focused approach is required where the safety of a whole food is being considered.

4. Information considered in developing this approach includes:

A) uses of living microorganisms in food production;

B) consideration of the types of genetic modifications likely to have been made in these organisms;

C) the types of methodologies available for performing a safety assessment; and

D) issues specific to the use of the recombinant-DNA microorganism in food production, including its genetic stability, potential for gene transfer, colonization of the gastrointestinal tract and persistence[4] therein,

---

[1] The microorganisms included in these applications are bacteria, yeasts, and filamentous fungi. (Such uses could include, but are not limited to, production of yogurt, cheese, fermented sausages, natto, kimchi, bread, beer, and wine.)

[2] The criterion for establishing the safety of microorganisms used in the production of foods where there is no history of safe use is beyond the scope of the current document.

[3] The Joint FAO/WHO Expert Committee on Food Additives (JECFA) is revising guidelines for General Specifications and Considerations for Enzyme Preparations used in food processing. These guidelines have been used to evaluate enzyme preparations derived from genetically modified microorganisms.

[4] Persistence connotes survival of microorganisms in the gastrointestinal tract longer than two intestinal transit times (International Life Science Institute, *The safety assessment of viable genetically modified microorganisms used as food,* 1999, Brussels; the Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology- *Safety assessment of foods derived from genetically modified microorganisms*, 24-28 September, 2001, Geneva, Switzerland).

interactions that the recombinant-DNA microorganism may have with the gastrointestinal flora or the mammalian host, and any impact of the recombinant-DNA microorganism on the immune system.

5. This approach is based on the principle that the safety of foods produced using recombinant-DNA microorganisms is assessed relative to the conventional counterparts that have a history of safe use, not only for the food produced using a recombinant-DNA microorganism, but also for the microorganism itself. This approach takes both intended and unintended effects into account. Rather than trying to identify every hazard associated with a particular food or the microorganism, the intention is to identify new or altered hazards relative to the conventional counterpart.

6. This safety assessment approach falls within the risk assessment framework as discussed in Section 3 of the Principles for the Risk Analysis of Foods Derived from Modern Biotechnology. If a new or altered hazard, nutritional or other food safety concern is identified by the safety assessment, the risk associated with it would first be assessed to determine its relevance to human health. Following the safety assessment and, if necessary, further risk assessment, the food or component of food, such as a microorganism used in production, would be subjected to risk management considerations in accordance with the Principles for the Risk Analysis of Foods Derived from Modern Biotechnology before it is considered for commercial distribution.

7. Risk management measures such as post-market monitoring of consumer health effects may assist the risk assessment process. These are discussed in paragraph 20 of the Principles for the Risk Analysis of Foods derived from Modern Biotechnology.

8. The Guideline describes approaches recommended for making safety assessments of foods produced using recombinant-DNA microorganisms, using comparison to a conventional counterpart. The safety assessment will focus on the safety of the recombinant-DNA microorganisms used in food production, and, where appropriate, on metabolites produced by the action of recombinant-DNA microorganisms on food. The Guideline identifies the data and information that are generally applicable to making such assessments. When conducting a comparison of a recombinant-DNA microorganism or a food produced using recombinant-DNA microorganism with their respective conventional counterparts, any identified differences should be taken into account, whether they are the result of intended or unintended effects. Due consideration should be given to the interactions of the recombinant-DNA microorganism with the food matrix or the microflora and to the safety of any newly-expressed protein(s) and secondary metabolic products. While this Guideline is designed for foods produced using recombinant-DNA microorganisms or their components, the approach described could, in general, be applied to foods produced using microorganisms that have been altered by other techniques.

## SECTION 2 – DEFINITIONS

9. The definitions below apply to this Guideline:

"***Recombinant-DNA Microorganism***" - means bacteria, yeasts or filamentous fungi in which the genetic material has been changed through *in vitro* nucleic acid techniques including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles.

"***Conventional Counterpart***"[5] – means:

- a microorganism/strain with a known history of safe use in producing and/or processing the food and related to the recombinant-DNA strain. The microorganism may be viable in the food or may be removed in processing or rendered non-viable during processing; or
- food produced using the traditional food production microorganisms for which there is experience of establishing safety based on common use in food production.

## SECTION 3 - INTRODUCTION TO FOOD SAFETY ASSESSMENT

10. Most foods produced as a result of the purposeful growth of microorganisms have their origins in antiquity, and have been deemed safe long before the emergence of scientific methods for assessing safety. Microorganisms possess properties, such as fast growth rates, that enable genetic modifications, whether employing conventional techniques or modern biotechnology, to be implemented in short time frames. Microorganisms used in food production derived using conventional genetic techniques have not customarily been systematically subjected to extensive chemical, toxicological, epidemiological, or medical evaluations prior to marketing. Instead microbiologists, mycologists, and food technologists have evaluated new strains of bacteria, yeasts and filamentous fungi for phenotypic characteristics that are useful in relation to food production.

11. Safety assessments of recombinant-DNA microorganisms should document the use of related microorganisms in foods, the absence of properties known to be characteristic of pathogens in the recombinant-DNA microorganisms or

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[5] It is recognized that for the foreseeable future, microorganisms derived from modern biotechnology will not be used as conventional counterparts.

the recipient strains used for constructing the recombinant-DNA microorganisms, and known adverse events involving the recipient or related organisms. In addition, when a recombinant DNA microorganism directly affects or remains in the food, any effects on the safety of the food should be examined.

12. The use of animal models for assessing toxicological effects is a major element in the risk assessment of many compounds, such as pesticides. In most cases, however, the substance to be tested is well characterized, of known purity, of no particular nutritional value, and human exposure to it is generally low. It is therefore relatively straightforward to feed such compounds to animals at a range of doses some several orders of magnitude greater than the expected human exposure levels, in order to identify any potential adverse health effects of importance to humans. In this way, it is possible, in most cases, to estimate levels of exposure at which adverse effects are not observed and to set safe intake levels by the application of appropriate safety factors.

13. Animal studies cannot readily be applied to testing the risks associated with whole foods, which are complex mixtures of compounds, and often characterized by a wide variation in composition and nutritional value. Due to their bulk and effect on satiety, they can usually only be fed to animals at low multiples of the amounts that might be present in the human diet. In addition, a key factor to consider in conducting animal studies on foods is the nutritional value and balance of the diets used, in order to avoid the induction of adverse effects that are not related directly to the material itself. Detecting any potential adverse effects and relating these conclusively to an individual characteristic of the food can therefore be extremely difficult. If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal studies could be requested on the whole food. Another consideration in deciding the need for animal studies is whether it is appropriate to subject experimental animals to such a study if it is unlikely to give rise to meaningful information.

14. Animal studies typically employed in toxicological evaluations also cannot be readily applied to testing potential risks associated with ingestion of microorganisms used for food production. Microorganisms are living entities, containing complex structures composed of many biochemicals, and therefore are not comparable to pure compounds. In some processed foods, they can survive processing and ingestion and can compete and, in some cases, be retained in the intestinal environment for significant periods of time. Appropriate animal studies should be used to evaluate the safety of recombinant-DNA microorganisms where the donor, or the gene or gene product do not have a history of safe use in food, taking into account available information regarding the donor and the characterization of the modified genetic material and the gene product. Further, appropriately designed studies in animals may be used to assess the nutritional value of the food or the bioavailability of the newly expressed substance in the food.

15. Due to the difficulties of applying traditional toxicological testing and risk assessment procedures to whole foods, a more focused approach is required for the safety assessment of foods produced using recombinant-DNA microorganisms. This has been addressed by the development of a multidisciplinary approach for assessing safety that takes into account the intended effect, the nature of the modification and detectable unintended changes that may occur in the microorganism or in its action on the food, using the concept of substantial equivalence[6].

16. While the focus of a safety assessment will be on the recombinant-DNA microorganism, additional information on its interaction with the food matrix should be taken into consideration when applying the concept of substantial equivalence, which is a key step in the safety assessment process. However, the concept of substantial equivalence is not a safety assessment in itself. Rather it represents the starting point that is used to structure the safety assessment of both a recombinant-DNA microorganism relative to its conventional counterpart and the food produced using recombinant-DNA microorganism relative to its conventional counterpart. This concept is used to identify for evaluation similarities and differences between a recombinant-DNA microorganisms used in food processing as well as the food produced using the recombinant-DNA microorganisms and their respective conventional counterparts as defined in paragraph 9. It aids in the identification of potential safety and nutritional issues and is considered the most appropriate strategy to date for safety assessment of foods produced using recombinant-DNA microorganisms. The safety assessment carried out in this way does not imply absolute safety of the new product; rather, it focuses on assessing the safety of any identified differences so that the safety of the recombinant-DNA microorganism and the food produced using recombinant-DNA microorganism can be considered relative to their respective conventional counterparts.

## UNINTENDED EFFECTS

17. In achieving the objective of conferring a specific target trait (intended effect) to a microorganism by the addition, substitution, removal, or rearrangement of defined DNA sequences, including those used for the purpose of DNA transfer or maintenance in the recipient organism, additional traits could, in some cases, be acquired or existing traits could be lost or modified. The potential for occurrence of unintended effects is not restricted to the use of *in vitro*

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[6] The concept of *substantial equivalence* as described in the Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology- Safety aspects of genetically modified plants, 29 May – 2 June, 2000, Geneva, Switzerland, and Section 4.3 of the Joint FAO/WHO Expert Consultation of Foods Derived from Biotechnology, - Safety assessment of foods derived from genetically modified microorganisms, 24-28 September, 2001, Geneva, Switzerland.

nucleic acid techniques. Rather, it is an inherent and general phenomenon that can also occur in the development of strains using traditional genetic techniques and procedures, or from exposure of microorganisms to intentional or unintended selective pressures. Unintended effects may be deleterious, beneficial, or neutral with respect to competition with other microorganisms, ecological fitness of the microorganism, the microorganism's effects on humans after ingestion, or the safety of foods produced using the microorganism. Unintended effects in recombinant-DNA microorganisms may also arise through intentional modification of DNA sequences or they may arise through recombination or other natural events in the recombinant-DNA microorganism. Safety assessment should include data and information to reduce the possibility that a food derived from a recombinant-DNA microorganism would have an unexpected, adverse effect on human health.

18. Unintended effects can result from the insertion of DNA sequences new to a microorganism into the microbial genome; they may be compared with those observed following the activity of naturally occurring transposable genetic elements. Insertion of DNA may lead to changes in expression of genes in the genome of the recipient. The insertion of DNA from heterologous sources into a gene may also result in the synthesis of a chimeric protein, also referred to as a fusion protein. In addition genetic instability and its consequences need to be considered.

19. Unintended effects may also result in the formation of new or changed patterns of metabolites. For example, the expression of enzymes at high levels or the expression of an enzyme new to the organism may give rise to secondary biochemical effects, changes in the regulation of metabolic pathways, or altered levels of metabolites.

20. Unintended effects due to genetic modification may be subdivided into two groups: those that could be predicted and those that are "unexpected." Many unintended effects are largely predictable based on knowledge of the added trait, its metabolic consequences or of the site of insertion. Due to the expanding knowledge of microbial genomes and physiology, and the increased specificity in function of genetic materials introduced through recombinant-DNA techniques compared with other forms of genetic manipulation, it may become easier to predict unintended effects of a particular modification. Molecular biological and biochemical techniques can also be used to analyse changes that occur at the level of transcription and translation that could lead to unintended effects.

21. The safety assessment of foods produced using recombinant-DNA microorganisms involves methods to identify and detect such unintended effects and procedures to evaluate their biological relevance and potential impact on food safety. A variety of data and information is necessary to assess unintended effects, because no individual test can detect all possible unintended effects or identify, with certainty, those relevant to human health. These data and information, when considered in total, should provide assurance that the food is unlikely to have an adverse effect on human health. The assessment of unintended effects takes into account the biochemical, and physiological characteristics of the microorganism that are typically selected for improving strains for commercial food or beverage uses. These determinations provide a first screen for microorganisms that exhibit unintended traits. Recombinant-DNA microorganisms that pass this screen are subjected to safety assessment as described in Section 4.

## FRAMEWORK OF FOOD SAFETY ASSESSMENT

22. The safety assessment of a food produced using a recombinant-DNA microorganism is based on determining the safety of using the microorganism, which follows a stepwise process of addressing relevant factors that include:

A) Description of the recombinant-DNA microorganism;

B) Description of the recipient microorganism and its use in food production;

C) Description of the donor organism(s);

D) Description of the genetic modification(s) including vector and construct;

E) Characterization of the genetic modification(s);

F) Safety assessment:

   a) expressed substances: assessment of potential toxicity and other traits related to pathogenicity;

   b) compositional analyses of key components;

   c) evaluation of metabolites;

   d) effects of food processing;

   e) assessment of immunological effects;

   f) assessment of viability and residence of microorganisms in the human gastrointestinal tract;

   g) antibiotic resistance and gene transfer; and

   h) nutritional modification.

23. In certain cases, the characteristics of the microorganisms and/or the foods produced/processed using these microorganisms may necessitate generation of additional data and information to address issues that are unique to the microorganisms and/or food products under review.

24. Experiments intended to develop data for safety assessments should be designed and conducted in accordance with sound scientific concepts and principles, as well as, where appropriate, Good Laboratory Practice. Primary data should be made available to regulatory authorities upon request. Data should be obtained using sound scientific methods and analysed using appropriate statistical techniques. The sensitivity of all analytical methods should be documented.

25. The goal of each safety assessment is to provide assurance, in the light of the best available scientific knowledge, that the food will not cause harm when prepared or consumed according to its intended use, nor should the organism itself cause harm when viable organisms remain in the food. Safety assessments should address the health aspects for the whole population, including immuno-compromised individuals, infants, and the elderly. The expected endpoint of such an assessment will be a conclusion regarding whether the new food and/or microorganisms are as safe as the conventional counterparts taking into account dietary impact of any changes in nutritional content or value. Where the microorganism is likely to be viable upon ingestion, its safety should be compared to a conventional counterpart taking into account residence of the recombinant-DNA microorganism in the gastrointestinal tract, and where appropriate, interactions between it and the gastrointestinal flora of mammals (especially humans) and impacts of the recombinant-DNA microorganism on the immune system. In essence, the outcome of the safety assessment process is to define the product under consideration in such a way as to enable risk managers to determine whether any measures are needed to protect the health of consumers and if so to make well-informed and appropriate decisions in this regard.

## SECTION 4- GENERAL CONSIDERATIONS

### DESCRIPTION OF THE RECOMBINANT-DNA MICROORGANISM

26. A description of the bacterial, yeast, or fungal strain and the food being presented for safety assessment should be provided. This description should be sufficient to aid in understanding the nature of the organism or food produced using the organism being submitted for safety assessment. Recombinant-DNA microorganisms used in food production or contained in food should be conserved as stock cultures with appropriate identification using molecular methods, and preferably, in established culture collections. This may facilitate the review of the original safety assessment. Such stock cultures should be made available to regulatory authorities upon request.

### DESCRIPTION OF THE RECIPIENT MICROORGANISM AND ITS USE IN FOOD PRODUCTION

27. A comprehensive description of the recipient microorganism or microorganism subjected to the modification should be provided. Recipient microorganisms should have a history of safe use in food production or safe consumption in foods. Organisms that produce toxins, antibiotics or other substances that should not be present in food, or that bear genetic elements that could lead to genetic instability, antibiotic resistance or that are likely to contain genes conferring functions associated with pathogenicity (i.e., also known as pathogenicity islands or virulence factors) should not be considered for use as recipients. The necessary data and information should include, but need not be restricted to:

A) identity: scientific name, common name or other name(s) used to reference the microorganism, strain designation, information about the strain and its source, or accession numbers or other information from a recognized culture repository from which the organism or its antecedents may be obtained, if applicable, information supporting its taxonomical assignment;

B) history of use and cultivation, known information about strain development (including isolation of mutations or antecedent strains used in strain construction); in particular, identifying traits that may adversely impact human health;

C) information on the recipient microorganism's genotype and phenotype relevant to its safety, including any known toxins, antibiotics, antibiotic resistance factors or other factors related to pathogenicity, or immunological impact, and information about the genetic stability of the microorganism;

D) history of safe use in food production or safe consumption in food; and

E) information on the relevant production parameters used to culture the recipient microorganism.

28. Relevant phenotypic and genotypic information should be provided not only for the recipient microorganism, but also for related species and for any extra-chromosomal genetic elements that contribute to the functions of the recipient strain, particularly if the related species are used in foods or involved in pathogenic effects in humans or other animals. Information on the genetic stability of the recipient microorganism should be considered including, as appropriate, the presence of mobile DNA elements, i.e. insertion sequences, transposons, plasmids, and prophages.

29. The history of use may include information on how the recipient microorganism is typically grown, transported and stored, quality assurance measures typically employed, including those to verify strain identity and production

specifications for microorganisms and foods, and whether these organisms remain viable in the processed food or are removed or rendered non-viable as a consequence of processing.

### DESCRIPTION OF THE DONOR ORGANISM(S)

30. Information should be provided on the donor organism(s) and any intermediate organisms, when applicable, and, when relevant, related organisms. It is particularly important to determine if the donor or intermediate organism(s) or other closely related species naturally exhibit characteristics of pathogenicity or toxin production, or have other traits that affect human health. The description of the donor or intermediate organism(s) should include:

A) identity: scientific name, common name or other name(s) used to reference the organism, strain designation, information about the strain and its source, or accession numbers or other information from a recognized culture repository from which the organism or its antecedents may be obtained, if applicable, and information supporting its taxonomic assignment;

B) information about the organism or related organisms that concerns food safety;

C) information on the organism's genotype and phenotype relevant to its safety including any known toxins, antibiotics, antibiotic resistance factors or other factors related to pathogenicity, or immunological impact; and

D) information on the past and present use, if any, in the food supply and exposure route(s) other than intended food use (e.g., possible presence as contaminants).

### DESCRIPTION OF THE GENETIC MODIFICATION(S) INCLUDING VECTOR AND CONSTRUCT

31. Sufficient information should be provided on the genetic modification(s) to allow for the identification of all genetic material potentially delivered to or modified in the recipient microorganism and to provide the necessary information for the analysis of the data supporting the characterization of the DNA added to, inserted into, modified in, or deleted from the microbial genome.

32. The description of the strain construction process should include:

A) information on the specific method(s) used for genetic modification;

B) information on the DNA used to modify the microorganism, including the source (e.g., plant, microbial, viral, synthetic), identity and expected function in the recombinant-DNA microorganism, and copy number for plasmids; and

C) intermediate recipient organisms including the organisms (e.g., other bacteria or fungi) used to produce or process DNA prior to introduction into the final recipient organism.

33. Information should be provided on the DNA added, inserted, deleted, or modified, including:

A) the characterization of all genetic components including marker genes, vector genes, regulatory and other elements affecting the function of the DNA;

B) the size and identity;

C) the location and orientation of the sequence in the final vector/construct; and

D) the function.

### CHARACTERIZATION OF THE GENETIC MODIFICATION(S)

34. In order to provide clear understanding of the impact of the genetic modification on the composition and safety of foods produced using recombinant-DNA microorganisms, a comprehensive molecular and biochemical characterization of the genetic modification should be carried out. To facilitate the safety assessment, the DNA to be inserted should be preferably limited to the sequences necessary to perform the intended functions.

35. Information should be provided on the DNA modifications in the recombinant DNA microorganism; this should include:

A) the characterization and description of the added, inserted, deleted, or otherwise modified genetic materials, including plasmids or other carrier DNA used to transfer desired genetic sequences. This should include an analysis of the potential for mobilization of any plasmids or other genetic elements used, the locations of the added, inserted, deleted, or otherwise modified genetic materials (site on a chromosomal or extra-chromosomal location); if located on a multi-copy plasmid, the copy number of the plasmid;

B) the number of insertion sites;

C) the organisation of the modified genetic material at each insertion site including the copy number and sequence data of the inserted, modified, or deleted material, plasmids or carrier DNA used to transfer the desired genetic

sequences, and the surrounding sequences. This will enable the identification of any substances expressed as a consequence of the inserted, modified or deleted material;

D) identification of any open reading frames within inserted DNA, or created by the modifications to contiguous DNA in the chromosome or in a plasmid, including those that could result in fusion proteins; and

E) particular reference to any sequences known to encode, or to influence the expression of, potentially harmful functions.

36. Information should be provided on any expressed substances in the recombinant-DNA microorganism; this should include:

A) the gene product(s) (e.g., a protein or an untranslated RNA) or other information such as analysis of transcripts or expression products to identify any new substances that may be present in the food;

B) the gene product's function;

C) the phenotypic description of the new trait(s);

D) the level and site of expression (intracellular, periplasmic - for Gram-negative bacteria, organellar - in eukaryotic microorganisms, secreted) in the microorganism of the expressed gene product(s), and, when applicable, the levels of its metabolites in the organism;

E) the amount of the inserted gene product(s) if the function of the expressed sequence(s)/gene(s) is to alter the level of a specific endogenous mRNA or protein; and

F) the absence of a gene product, or alterations in metabolites related to gene products, if applicable to the intended function(s) of the genetic modification(s).

37. In addition, information should be provided:

A) to demonstrate whether the arrangement of the modified genetic material has been conserved[7] or whether significant rearrangements have occurred after introduction to the cell and propagation of the recombinant strain to the extent needed for its use(s) in food production, including those that may occur during its storage according to current techniques;

B) to demonstrate whether deliberate modifications made to the amino acid sequence of the expressed protein result in changes in its post-translational modification or affect sites critical for its structure or function;

C) to demonstrate whether the intended effect of the modification has been achieved and that all expressed traits are expressed and inherited in a manner that is stable for the extent of propagation needed for its use(s) in food production and is consistent with laws of inheritance. It may be necessary to examine the inheritance of the inserted or modified DNA or the expression of the corresponding RNA if the phenotypic characteristics cannot be measured directly[8];

D) to demonstrate whether the newly expressed trait(s) is expressed as expected and targeted to the appropriate cellular location or is secreted in a manner and at levels that is consistent with the associated regulatory sequences driving the expression of the corresponding gene;

E) to indicate whether there is any evidence to suggest that one or more genes in the recipient microorganism has been affected by the modifications or the genetic exchange process; and

F) to confirm the identity and expression pattern of any new fusion proteins.

## SAFETY ASSESSMENT

38. The safety assessment of the modified microorganism should be performed on a case by case basis depending on the nature and extent of the introduced changes. Conventional toxicology studies may not be considered necessary where the substance or a closely related substance has, taking into account its function and exposure, been consumed safely in food. In other cases, the use of appropriate conventional toxicology or other studies on the new substance may be necessary. Effects of the recombinant-DNA microorganism on the food matrix should be considered as well. If the characterisation of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal or *in vitro* studies with the recombinant-DNA microorganism and/or the food produced using it could be considered necessary.

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[7] Microbial genomes are more fluid than those of higher eukaryotes; that is, the organisms grow faster, adapt of changing environments, and are more prone to change. Chromosomal rearrangements are common. The general genetic plasticity of microorganisms may affect recombinant DNA in microorganisms and must be considered in evaluating the stability of recombinant DNA microorganisms.

[8] Modified strains should be maintained in a manner to enable verification of the genetic stability.

## Expressed Substances: Assessment of Potential Toxicity and Other Traits Related to Pathogenicity

39. When a substance is new to foods or food processing, the use of conventional toxicology studies or other applicable studies on the new substance will be necessary. This may require the isolation of the new substance from the recombinant-DNA microorganism, the food product if the substance is secreted, or, if necessary, the synthesis or production of the substance from an alternative source, in which case the material should be shown to be structurally, functionally, and biochemically equivalent to that produced in the recombinant-DNA microorganism. Information on the anticipated exposure of consumers to the substance, the potential intake and dietary impact of the substance should be provided.

40. The safety assessment of the expressed substance should take into account its function and concentration in the food. The number of viable microorganisms remaining in the food should be also determined and compared to a conventional counterpart. All quantitative measurements should be analysed using appropriate statistical techniques. Current dietary exposure and possible effects on population sub-groups should also be considered.

- In the case of proteins, the assessment of potential toxicity should take into account the structure and function of the protein and should focus on amino acid sequence similarity between the protein and known protein toxins and anti-nutrients (e.g., protease inhibitors, siderophores) as well as stability to heat or processing and to degradation in appropriate representative gastric and intestinal model systems. Appropriate oral toxicity studies[9] may be carried out in cases where the protein is present in the food, but is not closely similar to proteins that have been safely consumed in food, and has not previously been consumed safely in food, and taking into account its biological function in microorganisms where known.

- Potential toxicity of non-protein substances that have not been safely consumed in food should be assessed in a case-by-case basis depending on the identity, concentration, and biological function of the substance and dietary exposure. The type of studies to be performed may include evaluations of metabolism, toxicokinetics, chronic toxicity/ carcinogenicity, impact on reproductive function, and teratogenicity.

41. The newly expressed or altered properties should be shown to be unrelated to any characteristics of donor organisms that could be harmful to human health. Information should be provided to ensure that genes coding for known toxins or anti-nutrients present in the donor organisms are not transferred to recombinant-DNA microorganisms that do not normally express those toxic or anti-nutritious characteristics.

- Additional *in vivo* or *in vitro* studies may be needed on a case-by-case basis to assess the toxicity of expressed substances, taking into account the potential accumulation of any substances, toxic metabolites or antibiotics that might result from the genetic modification.

## Compositional Analyses of Key Components

42. Analyses of concentrations of key components[10] of foods produced by recombinant-DNA microorganisms should be compared with an equivalent analysis of a conventional counterpart produced under the same conditions. The statistical significance of any observed differences should be assessed in the context of the range of natural variations for that parameter to determine its biological significance. Ideally, the comparator(s) used in this assessment should be food produced using the near isogenic parent strain. The purpose of this comparison, in conjunction with an exposure assessment as necessary, is to establish that substances that can affect the safety of the food have not been altered in a manner that would have an adverse impact on human health.

## Evaluation of Metabolites

43. Some recombinant-DNA microorganisms may be modified in a manner that could result in new or altered levels of various metabolites in foods produced using these organisms. Where altered metabolite levels are identified in foods, consideration should be given to the potential impacts on human health using conventional procedures for establishing the safety of such metabolites (e.g., procedures for assessing the human safety of chemicals in foods).

44. New or altered levels of metabolites produced by a recombinant-DNA microorganism may change the population of microorganisms in mixed culture, potentially increasing the risk for growth of harmful organisms or accumulation of harmful substances. Possible effects of genetic modification of a microorganism on other microorganisms should be

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[9] Guidelines for oral toxicity studies have been developed in international fora, for example the OECD Guidelines for the Testing of Chemicals.

[10] Key nutrients or key anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major nutritional constituents (fats, proteins, carbohydrates), enzyme inhibitors as anti-nutrients, or minor compounds (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be produced by the microorganism, such as those compounds whose toxic potency and level may be significant to health. Microorganisms traditionally used in food processing are not usually known to produce such compounds under production conditions.

assessed when a mixed culture of microorganisms is used for food processing, such as for production of natural cheese, miso, soy sauce, etc.

### Effects of Food Processing

45. The potential effects of food processing, including home preparation, on foods produced using recombinant-DNA microorganisms should also be considered. For example, alterations could occur in the heat stability of an endogenous toxicant or the bioavailability of an important nutrient after processing. Information should therefore be provided describing the processing conditions used in the production of a food. For example, in the case of yoghurt, information should be provided on the growth of the organism and culture conditions.

### Assessment of Immunological Effects

46. When the protein(s) resulting from an inserted gene is present in the food, it should be assessed for its potential to cause allergy. The likelihood that individuals may already be sensitive to the protein and whether a protein new to the food supply will induce allergic reactions should be considered. A detailed presentation of issues to be considered is presented in the Annex to this guideline.

47. Genes derived from known allergenic sources should be assumed to encode an allergen and be avoided unless scientific evidence demonstrates otherwise. The transfer of genes from organisms known to elicit gluten-sensitive enteropathy in sensitive individuals should be avoided unless it is documented that the transferred gene does not code for an allergen or for a protein involved in gluten-sensitive enteropathy.

48. Recombinant-DNA microorganisms that remain viable in foods may interact with the immune system in the gastrointestinal tract. Closer examination of these interactions will depend on the types of differences between the recombinant-DNA microorganism and its conventional counterpart.

### Assessment of Viability and Residence of Microorganisms in the Human Gastrointestinal Tract

49. In some foods produced using recombinant-DNA microorganisms, ingestion of these microorganisms and their residence[11] may have an impact on the human intestinal tract. The need for further testing of such microorganisms should be based on the presence of their conventional counterpart in foods, and the nature of the intended and unintended effects of genetic modifications. If processing of the final food product eliminates viable microorganisms (by heat treatment in baking bread, for example), or if accumulations of end-products toxic to the microorganism (such as alcohol or acids) eliminate viability, then viability and residence of microorganisms in the alimentary system need no examination.

50. For applications in which recombinant-DNA microorganisms used in production remain viable in the final food product, (for example, organisms in some dairy products), it may be desirable to demonstrate the viability (or residence time) of the microorganism alone and within the respective food matrix in the digestive tract and the impact on the intestinal microflora in appropriate systems. The nature of intended and unintended effects of genetic modification and the degree of differences from the conventional counterpart will determine the extent of such testing.

### ANTIBIOTIC RESISTANCE AND GENE TRANSFER

51. In general, traditional strains of microorganisms developed for food processing uses have not been assessed for antibiotic resistance. Many microorganisms used in food production possess intrinsic resistance to specific antibiotics. Such properties need not exclude such strains from consideration as recipients in constructing recombinant-DNA microorganisms. However, strains in which antibiotic resistance is encoded by transmissible genetic elements should not be used where such strains or these genetic elements are present in the final food. Any indication of the presence of plasmids, transposons, and integrons containing such resistance genes should be specifically addressed.

52. Alternative technologies, demonstrated to be safe, that do not rely on antibiotic resistance marker genes in viable microorganisms present in foods should be used for selection purposes in recombinant-DNA microorganisms. In general, use of antibiotic resistance markers for constructing intermediate strains should pose no significant hazards that would exclude the use of the ultimate strains in food production, provided that the antibiotic resistance marker genes have been removed from the final construct.

53. Transfer of plasmids and genes between the resident intestinal microflora and ingested recombinant-DNA microorganisms may occur. The possibility and consequences of gene transfer from recombinant-DNA microorganisms and food products produced by recombinant-DNA microorganisms to gut microorganisms or human cells should also

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[11] Permanent life-long colonization by ingested microorganisms is rare. Some orally administered microorganisms have been recovered in faeces or in the colonic mucosa weeks after feeding ceased. Whether the genetically modified microorganism is established in the gastrointestinal tract or not, the possibility remains that it might influence the microflora or the mammalian host (Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology – *Safety assessment of foods derived from genetically modified microorganism*, 24-28 September, 2001, Geneva, Switzerland).

be considered. Transferred DNA would be unlikely to be maintained in the absence of selective pressure. Nevertheless, the possibility of such events cannot be completely discounted.

54. In order to minimize the possibility of gene transfer, the following steps should be considered:

A) chromosomal integration of the inserted genetic material may be preferable to localization on a plasmid;

B) where the recombinant-DNA microorganism will remain viable in the gastrointestinal tract, genes should be avoided in the genetic construct that could provide a selective advantage to recipient organisms to which the genetic material is unintentionally transferred; and

C) sequences that mediate integration into other genomes should be avoided in constructing the introduced genetic material.

### NUTRITIONAL MODIFICATION

55. The assessment of possible compositional changes to key nutrients, which should be conducted for all foods produced using recombinant-DNA microorganisms, has already been addressed under 'Compositional analyses of key components.' If such nutritional modifications have been implemented, the food should be subjected to additional testing to assess the consequences of the changes and whether the nutrient intakes are likely to be altered by the introduction of such foods into the food supply.

56. Information about the known patterns of use and consumption of a food and its derivatives should be used to estimate the likely intake of the food produced using the recombinant-DNA microorganism. The expected intake of the food should be used to assess the nutritional implications of the altered nutrient profile both at customary and maximal levels of consumption. Basing the estimate on the highest likely consumption provides assurance that the potential for any undesirable nutritional effects will be detected. Attention should be paid to the particular physiological characteristics and metabolic requirements of specific population groups such as infants, children, pregnant and lactating women, the elderly and those with chronic diseases or compromised immune systems. Based on the analysis of nutritional impacts and the dietary needs of specific population subgroups, additional nutritional assessments may be necessary. It is also important to ascertain to what extent the modified nutrient is bioavailable and remains stable with time, processing, and storage.

57. The use of modern biotechnology to change nutrient levels in foods produced using microorganisms could result in broad changes to the nutrient profile. The intended modification in the microorganism could alter the overall nutrient profile of the product, which, in turn, could affect the nutritional status of individuals consuming the food. The impact of changes that could affect the overall nutrient profile should be determined.

58. When the modification results in a food product with a composition that is significantly different from its conventional counterpart, it may be appropriate to use additional conventional foods or food components (i.e., foods whose nutritional composition is closer to that of the food produced using the recombinant-DNA microorganism) as appropriate comparators to assess the nutritional impact of the food.

59. Some foods may require additional testing. For example, animal-feeding studies may be warranted for foods produced using recombinant-DNA microorganisms if changes in the bioavailability of nutrients are expected or if the composition is not comparable to conventional foods. Also, foods designed for health benefits, may require an assessment beyond the scope of these guidelines such as specific nutritional, toxicological or other appropriate studies. If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal studies could be requested on the whole food.

### REVIEW OF SAFETY ASSESSMENTS

60. The goal of the safety assessment is a conclusion as to whether the food produced using a recombinant-DNA microorganism is as safe as the conventional counterpart taking into account dietary impact of any changes in nutritional content or value. Nevertheless, the safety assessment should be reviewed in the light of new scientific information that calls into question the conclusions of the original safety assessment.

## ANNEX: ASSESSMENT OF POSSIBLE ALLERGENICITY

## SECTION 1 – INTRODUCTION

1. All newly expressed proteins[12] produced by recombinant-DNA microorganisms that could be present in the final food should be assessed for their potential to cause allergic reactions. This should include consideration of whether a newly expressed protein is one to which certain individuals may already be sensitive as well as whether a protein new to the food supply is likely to induce allergic reactions in some individuals.

2. At present, there is no definitive test that can be relied upon to predict allergic response in humans to a newly expressed protein, therefore, it is recommended that an integrated, stepwise, case by case approach, as described below, be used in the assessment of possible allergenicity of newly expressed proteins. This approach takes into account the evidence derived from several types of information and data since no single criterion is sufficiently predictive.

3. The endpoint of the assessment is a conclusion as to the likelihood of the protein being a food allergen.

## SECTION 2 - ASSESSMENT STRATEGY

4. The initial steps in assessing possible allergenicity of any newly expressed proteins are the determination of: the source of the introduced protein; any significant similarity between the amino acid sequence of the protein and that of known allergens; and its structural properties, including but not limited to, its susceptibility to enzymatic degradation, heat stability and/or, acid and enzymatic treatment.

5. As there is no single test that can predict the likely human IgE response to oral exposure, the first step to characterize newly expressed proteins should be the comparison of the amino acid sequence and certain physicochemical characteristics of the newly expressed protein with those of established allergens in a weight of evidence approach. This will require the isolation of any newly expressed proteins produced by recombinant-DNA microorganisms, or the synthesis or production of the substance from an alternative source, in which case the material should be shown to be structurally, functionally and biochemically equivalent to that produced by recombinant-DNA microorganisms. Particular attention should be given to the choice of the expression host, since post-translational modifications allowed by different hosts (i.e.: eukaryotic vs. prokaryotic systems) may have an impact on the allergenic potential of the protein.

6. It is important to establish whether the source is known to cause allergic reactions. Genes derived from known allergenic sources should be assumed to encode an allergen unless scientific evidence demonstrates otherwise.

## SECTION 3 – INITIAL ASSESSMENT

### SECTION 3.1 - SOURCE OF THE PROTEIN

7. As part of the data supporting the safety of foods produced using recombinant-DNA microorganisms, information should describe any reports of allergenicity associated with the donor organism. Allergenic sources of genes would be defined as those organisms for which reasonable evidence of IgE mediated oral, respiratory or contact allergy is available. Knowledge of the source of the introduced protein allows the identification of tools and relevant data to be considered in the allergenicity assessment. These include: the availability of sera for screening purposes; documented type, severity and frequency of allergic reactions; structural characteristics and amino acid sequence; physicochemical and immunological properties (when available) of known allergenic proteins from that source.

### SECTION 3.2 – AMINO ACID SEQUENCE HOMOLOGY

8. The purpose of a sequence homology comparison is to assess the extent to which a newly expressed protein is similar in structure to a known allergen. This information may suggest whether that protein has an allergenic potential. Sequence homology searches comparing the structure of all newly expressed proteins with all known allergens should be done. Searches should be conducted using various algorithms such as FASTA or BLASTP to predict overall structural similarities. Strategies such as stepwise contiguous identical amino acid segment searches may also be performed for identifying sequences that may represent linear epitopes. The size of the contiguous amino acid search should be based on a scientifically justified rationale in order to minimize the potential for false negative or false

[12] This assessment strategy is not applicable for assessing whether newly expressed proteins are capable of inducing gluten-sensitive or other enteropathies. The issue of enteropathies is already addressed in Assessment of immunological effects, paragraph 47 of the Guideline for the Conduct of Food Safety Assessment of Foods Produced using Recombinant-DNA Microorganisms. In addition, the strategy is not applicable to the evaluation of foods where gene products are down regulated for hypoallergenic purposes.

positive results[13]. Validated search and evaluation procedures should be used in order to produce biologically meaningful results.

9. IgE cross-reactivity between the newly expressed protein and a known allergen should be considered a possibility when there is more than 35% identity in a segment of 80 or more amino acids (FAO/WHO 2001) or other scientifically justified criteria. All the information resulting from the sequence homology comparison between the newly expressed protein and known allergens should be reported to allow a case-by-case scientifically based evaluation.

10. Sequence homology searches have certain limitations. In particular, comparisons are limited to the sequences of known allergens in publicly available databases and the scientific literature. There are also limitations in the ability of such comparisons to detect non-contiguous epitopes capable of binding themselves specifically with IgE antibodies.

11. A negative sequence homology result indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens. A result indicating absence of significant sequence homology should be considered along with the other data outlined under this strategy in assessing the allergenic potential of newly expressed proteins. Further studies should be conducted as appropriate (see also sections 4 and 5). A positive sequence homology result indicates that the newly expressed protein is likely to be allergenic. If the product is to be considered further, it should be assessed using serum from individuals sensitized to the identified allergenic source.

### SECTION 3.3 – PEPSIN RESISTANCE

12. Resistance to pepsin digestion has been observed in several food allergens; thus a correlation exists between resistance to digestion by pepsin and allergenic potential[14]. Therefore, the resistance of a protein to degradation in the presence of pepsin under appropriate conditions indicates that further analysis should be conducted to determine the likelihood of the newly expressed protein being allergenic. The establishment of a consistent and well-validated pepsin degradation protocol may enhance the utility of this method. However, it should be taken into account that a lack of resistance to pepsin does not exclude that the newly expressed protein can be a relevant allergen.

13. Although the pepsin resistance protocol is strongly recommended, it is recognized that other enzyme susceptibility protocols exist. Alternative protocols may be used where adequate justification is provided[15].

## SECTION 4 – SPECIFIC SERUM SCREENING

14. For those proteins that originate from a source known to be allergenic, or have sequence homology with a known allergen, testing in immunological assays should be performed where sera are available. Sera from individuals with a clinically validated allergy to the source of the protein can be used to test the specific binding to IgE class antibodies of the protein in *in vitro* assays. A critical issue for testing will be the availability of human sera from sufficient numbers of individuals[16]. In addition, the quality of the sera and the assay procedure need to be standardized to produce a valid test result. For proteins from sources not known to be allergenic, and which do not exhibit sequence homology to a known allergen, targeted serum screening may be considered where such tests are available as described in paragraph 17.

15. In the case of a newly expressed protein derived from a known allergenic source, a negative result in *in vitro* immunoassays may not be considered sufficient, but should prompt additional testing, such as the possible use of skin test and *ex vivo* protocols[17]. A positive result in such tests would indicate to a potential allergen.

## SECTION 5 – OTHER CONSIDERATIONS

16. The absolute exposure to the newly expressed protein and the effects of relevant food processing will contribute toward an overall conclusion about the potential for human health risk. In this regard, the nature of the food product intended for consumption should be taken into consideration in determining the types of processing which would be applied and its effects on the presence of the protein in the final food product.

---

13 It is recognized that the 2001 FAO/WHO consultation suggested moving from 8 to 6 identical amino acid segment searches. The smaller the peptide sequence used in the stepwise comparison, the greater the likelihood of identifying false positives, inversely, the larger the peptide sequence used, the greater the likelihood of false negatives, thereby reducing the utility of the comparison.

14 The method outlined in the U.S. Pharmacopoeia (1995) was used in the establishment of the correlation (Astwood *et al.*, 1996).

15 Reference to Joint FAO/WHO Expert Consultation (2001).

16 According to the Report of the Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (22-25 January 2001, Rome, Italy) a minimum of 8 relevant sera is required to achieve a 99% certainty that the new protein is not an allergen in the case of a major allergen. Similarly, a minimum of 24 relevant sera is required to achieve the same level of certainty in the case of a minor allergen. It is recognized that these quantities of sera may not be available for testing purposes.

17 Reference to Joint FAO/WHO Expert Consultation (2001) on description of *ex vivo*.

17. As scientific knowledge and technology evolves, other methods and tools may be considered in assessing the allergenicity potential of newly expressed proteins as part of the assessment strategy. These methods should be scientifically sound and may include targeted serum screening (i.e. the assessment of binding to IgE in sera of individuals with clinically validated allergic responses to broadly-related categories of foods); the development of international serum banks; use of animal models; and examination of newly expressed proteins for T-cell epitopes and structural motifs associated with allergens.

# ANNEX E

Five pages have been removed in accordance with copyright laws. The removed reference citation is:

United States Pharmacopeial Convention, 2020, Food Chemicals Codex (FCC) 12, p. 404, Enzyme Preparations. Washington (DC).

# ANNEX F

Eleven pages have been removed in accordance with copyright laws. The removed reference citation is:

D. Somashekar, R. Joseph, Chitosanases – Properties and Applications: A Review, Bioresource Technology, 55 (1996), 35-45.

# ANNEX G

Eleven pages have been removed. This publication is freely available at https://openaccesspub.org/tissue-repair-and-regeneration/article/657 (accessed 2023-12-11).

A. Lončarević, M. Ivanković, and A. Rogina, Lysozyme-Induced Degradation of Chitosan: The Characterisation of Degraded Chitosan Scaffolds, Journal of Tissue Repair and Regeneration, vol. 1, Issue 1, pp. 12–22, 2017.

# 1. Title: Pepsin Digestion Study of Chitosanase (EC 3.2.1.32)

**20th March, 2015**



| | |
|---|---|
| | |
| **Study Director** | **Study Monitor** |
| **Dr. Anil K. Gupta**<br>Advanced Enzyme Technologies Ltd.<br>A 61/62 Malegaon MIDC, Sinnar<br>Nashik-422113 India.<br>Email: anil@advancedenzymes.com | **Dr. Stephanie A. Misquitta**<br>Advanced Enzyme Technologies Ltd.<br>“A” Wing, Sun Magnetica, 5th Floor,<br>LIC Service Rd., Louiswadi,<br>Thane (W), Pin 400 604, Maharashtra, India.<br>Email: stephanie@advancedenzymes.com |

## *2. Table of Contents*

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### *3. Rationale*

Proteins that are susceptible to digestion by pepsin are less likely to elicit an allergenic response. Hence the digestibility of the "test protein' by pepsin, was evaluated *in vitro* by exposing it to the action of pepsin in the presence of simulated gastric fluid (SGF).

The pepsin digestion study is based on the recommendations made by the Joint FAO/WHO Expert Consultation, 2001[1]. The protocol used for this assay is based on the detailed study undertaken by Thomas *et al*[2], which in turn takes into consideration the guidelines provided by the Codex Alimentarius Commission, 2003[3,7,8]. The same study has been referred to, in the EFSA Journal[4].

### *4. Method*

Enzyme sample details:

Product name : Chitosanase

Batch No. : 061434

Manufactured : 06/2014

The pepsin digestion reaction was performed by subjecting the protein to the action of pepsin by mixing the protein in stimulated gastric fluid (SGF), containing pepsin. Briefly, the assay was performed at a pH of 1.2 and temperature, 37°C, using 10 units of pepsin activity, per microgram of 'test protein'. For comparison, control reactions were set up:
(i) 'test protein' without pepsin, to evaluate the stability of the 'test protein' in the absence of pepsin and (ii) pepsin alone, to evaluate auto-digestion. Samples were removed at specific times and the digestion reaction quenched, by neutralization with a carbonate buffer containing the SDS-PAGE sample loading buffer. The samples were analysed by SDS-PAGE and stained with Coomassie blue to evaluate the efficiency of digestion. Following destaining of the gels, the gels were assessed for the "time to disappearance", ie: the time point at which the full length protein or fragment is not detected.

To validate the method used in our study, the pepsin digestion reaction was also carried out on two proteins, beta lactoglobulin and acid phosphatase. These two proteins are recommended to be used as comparators[1]. These proteins have earlier been studied for pepsin resistance. Beta lactoglobulin is considered to be a major allergen. It is stable and not digested by pepsin[2], hence it can be detected even after 60 minutes of incubation with pepsin. However, acid phosphatase is not stable and easily digested by pepsin[5].

## *5. Results*

When chitosanase is exposed to pepsin, it is completely digested in 0.5 minutes (Fig. 1A, Lane 2).

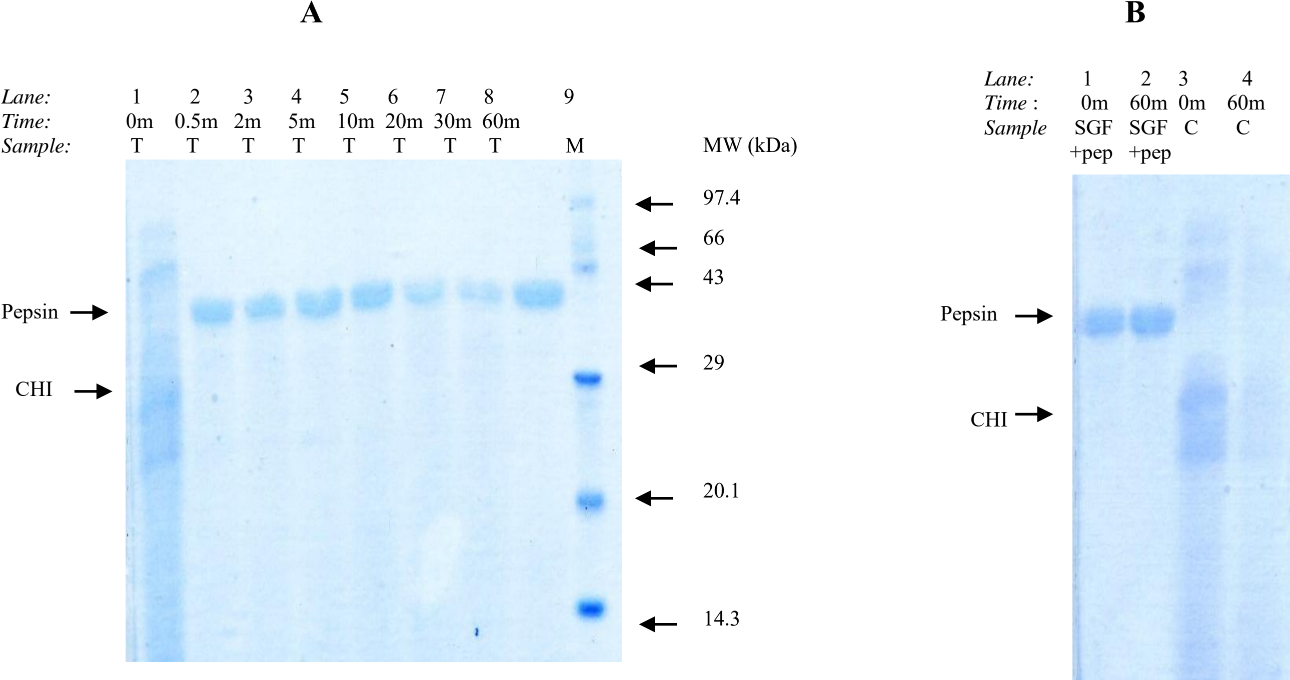


**Figure 1:** Time dependent digestion of Chitosanase (CHI) by pepsin, analysed by SDS-PAGE

(A) *Test:*
Lanes 1-8: Chitosanase in SGF (*with pepsin*) at time points 0, 0.5, 2, 5, 10, 20, 30 and 60 minutes respectively.
Lane 9: M, Protein standards.

(B) *Control:*
Lanes 1 and 2: SGF with pepsin alone (0 and 60 minutes respectively);
Lanes 3 and 4: Control reaction, Chitosanase in SGF (*without pepsin*) (0 and 60 minutes respectively).

CHI: Chitosanase, pep: pepsin, M: Molecular weight standards, C: Control Reaction , T: Test Reaction

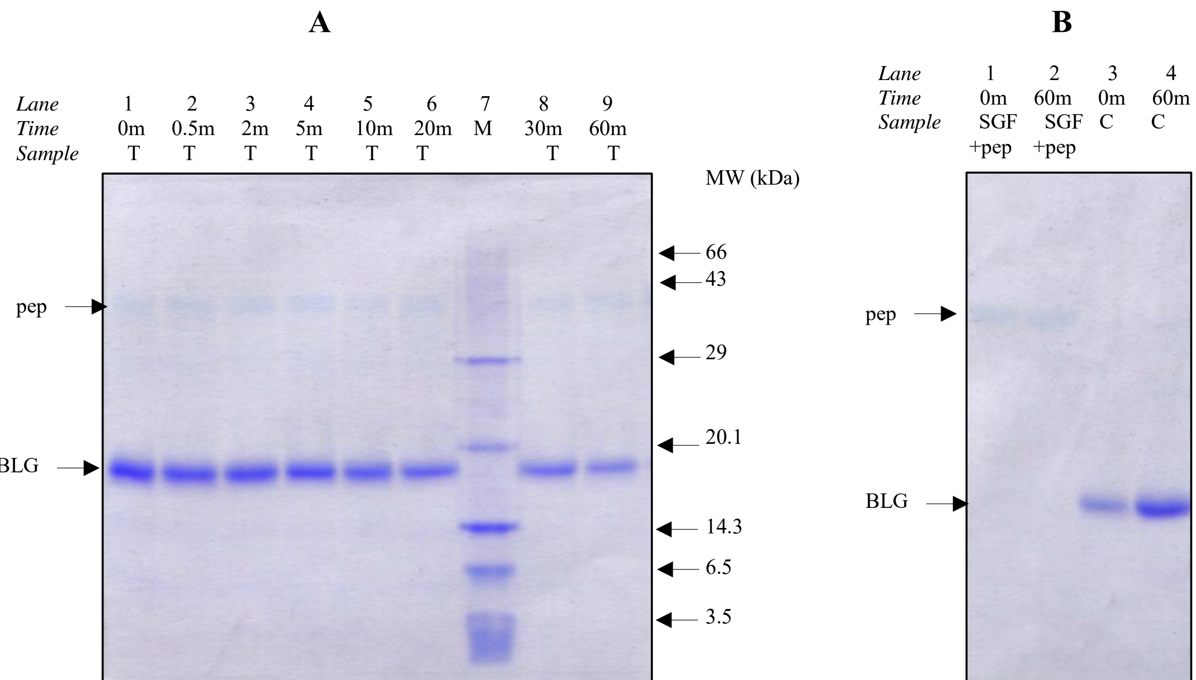


**Figure 2:** Time dependent digestion of beta-lactoglobulin (BLG), by pepsin, analysed by SDS-PAGE

**(A):** *Test*
Lane 1-6 and Lane 8--9: Beta lactoglobulin in SGF (with pepsin) at time points 0, 0.5, 2, 5, 10, 20, 30 and 60 minutes respectively.
Lane 7: M, Protein standards.

**(B):** *Control*
Lanes 1 and 2: SGF with pepsin alone (0 and 60 minutes respectively);
Lanes 3 and 4: Control reaction, Beta lactoglobulin in SGF (without pepsin) (0 and 60 minutes respectively).

BLG: Beta lactoglobulin, pep: pepsin, M: Molecular weight standards, C: Control Reaction (no pepsin), T: Test Reaction (with pepsin)

As evident from Fig. 2, beta-lactoglobulin is not digested even by the end of 60 minutes. These results for beta-lactoglobulin are similar to that obtained by Thomas *et al*[2], and serves as a validation of the method used in our study.

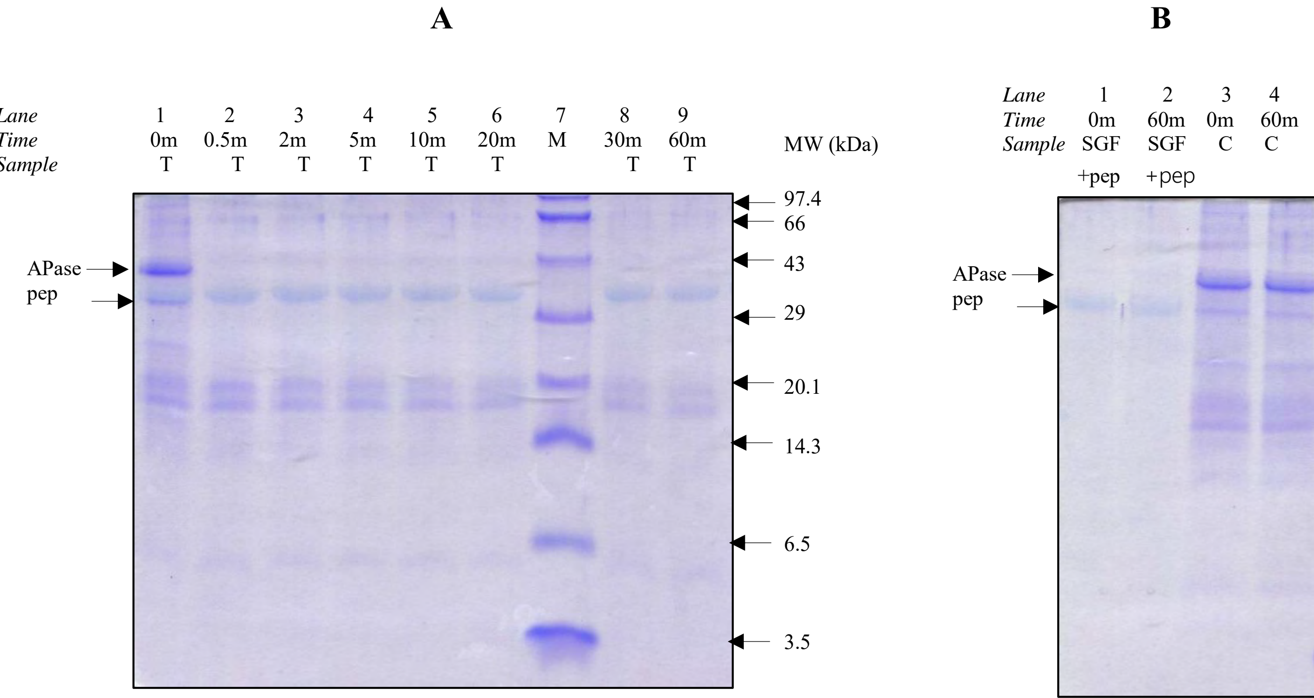


**Figure 3:** Time dependent digestion of acid phosphatase (APase), by pepsin, analysed by SDS-PAGE.

**(A):** *Test:*
Lane 1-6 and Lane 8--9: Acid phosphatase in SGF (with pepsin) at time points 0, 0.5, 2, 5, 10, 20, 30 and 60 minutes respectively.
Lane 7: M, Protein standards.

**(B):** *Controls:*
Lanes 1 and 2: SGF with pepsin alone (0 and 60 minutes respectively);
Lanes 3 and 4: Control reaction, Acid phosphatase in SGF (without pepsin) (0 and 60 minutes respectively).

APase: Acid phosphatase, pep: pepsin, M: Molecular weight standards, C: Control, T: Test

As evident from Fig. 3, acid phosphatase (APase) is not visible at the end of 0.5 minutes, indicating that it is digested during this time. No protein fragments are generated. These results for acid phosphatase are similar to that obtained by Astwood *et al*[5], and serves as a validation of the method used in our study.

## *6. Discussion*

Chitosanase protein gets digested by pepsin, within 30 seconds and is unlikely to elicit allergenicity.

## *7. Conclusion*

Results from this study show that chitosanase can be considered to be a labile protein, as it is digested by pepsin in 30 seconds, and therefore is not likely to be allergenic.

## *8. Bibliography*

1. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology. (2001). *Evaluation of Allergenicity of Genetically Modified Foods.* Rome, Italy: Food and Agriculture Organization of the United Nations (FAO).

2. Thomas, K., Aalbers, M., Bannon, G., Bartels, M., Dearman, R., Esdaile, D., et al. (2004). A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins. *Regulatory Toxicology and Pharmacology*, 39: pp. 87-98.

3. Codex Alimentarius (2003). Codex Alinorm 03/34: Joint FAO/WHO Food Standard Programme. (Twenty Fifth Session, Rome 2003). Appendix III, Guideline for the conduct of food safety assessment of foods derived from recombinant -DNA plants, and Appendix IV, Annex on the assessment of possible allergenicity., (pp. pp. 47-60). Rome.

4. EFSA Panel on Genetically Modified Organisms (GMO). (2010). Draft Scientific Opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed. *EFSA Journal*, 8 (7):1700 [168 pp.].

5. Astwood, J., Leach, J., & Fuchs, R. (1996). Stability of food allergens to digestion invitro. *NATURE BIOTECHNOLOGY*, Vol. 14, pp. 1269-1273.

6. Board of Trustees ed. (1995). Simulated Gastric Fluid TS. *in The United States Pharmacopeia 23, The National Formulary 18. United States Pharmacopeial Convention, Inc., Rockville, MD.*, pp. 2053.

7. Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants (CAC/GL 45-2003), Annexes II and III adopted in 2008.

8. Guideline for the conduct of Food Safety assessment of foods produced using recombinant-DNA microorganisms (CAC/GL 46-2003).

***Abbreviations:***
kDa: kiloDaltons
SGF: Simulated Gastric Fluid
SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

# ANNEX I

Title: Sequence: Allergenicity analysis for Chitosanase from *Bacillus subtilis* strain CSSC
AETL/AML1/GD-UP/026
Date: 15.10.2019

---

1. Title: Allergenicity analysis for chitosanase from *Bacillus subtilis* strain CSSC

2. Objective

   To assess the allergenicity of Chitosanase from *Bacillus subtilis* strain CSSC

3. Methodology

   In order to assess the allergenicity of the protein under scrutiny, sequence specific homology searches were conducted in the Structural Database of Allergenic Proteins (SDAP) (https://fermi.utmb.edu/SDAP/sdap_who.html)

4. Results

   Amino acid sequence of chitosanase from *Bacillus subtilis* strain CSSC

```
>lcl|CP031129_prot_DUT89_13605_2662 [gene=DUT89_13605]
[protein=chitosanase] [protein_id=DUT89_13605]
[location=complement(2582070..2582903)]

MKISMQKADFWKKAAISLLVFTMFFTLMMSETVFAAGLNKDQKRRAEQLTSIFENGTTEIQYGYVERLDDGRGY
TCGRAGFTTATGDALEVVEVYTKAVPNNKLKKYLPELRRLAKEESDDTSNLKGFASAWKSLANDKEFRAAQDKV
NDHLYYQPAMKRSDNAGLKTALARAVMYDTVIQHGDGDDPDSFYALIKRTNKKAGGSPKDGIDEKKWLNKFLDV
RYDDLMNPANHDTRDEWRESVARVDVLRSIAKENNYNLNGPIHVRSNEYGNFVIK
```

   A snapshot of the results obtained following homology sequence searches in SDAP database is presented below:

   **In case of an 80 amino acids sliding window**
   Chitosanase produced from *Bacillus subtilis* strain CSSC, did not show homology above the cutoff 35.00%, when searched in SDAP database.

   **In case of a full length FASTA alignment**
   Chitosanase produced from *Bacillus subtilis* strain CSSC, did not show match with any allergen when searched in SDAP database.

   **In case of peptide match of complete identity over 6 and 8 contiguous amino acids**
   No match with any known allergen was observed in case of chitosanase produced from *Bacillus subtilis* strain CSSC in SDAP database.

5. Conclusion

   Chitosanase sequence when analysed using SDAP online tool, did not show match with any known allergen.

6. AETL_AML1_GD-UP_026A_Chitosanase_Bs PLSSC_Allergenicity_151019: Detailed analysis report (SDAP data)

**Report code:** AR-20-IR-020763-03

**Batch code:** EUINBA-00064847

**Report date:** 02.05.2020

**Advanced Enzyme Technologies Ltd. - Thane (w)**
**A-135, Road No. 23,**
**Wagle Industrial estate**
**400604 Thane (w).**
**INDIA**

**Mr Chiranjit Maity**

# ANALYTICAL REPORT

| | | | |
|---|---|---|---|
| **Sample code:** | 258-2020-03005240 | **Received on:** | 20.03.2020 |
| **Sample name:** | Bacillus subtilis CSSC | **Analysed between:** | 24.03.2020 - 07.04.2020 |
| **Quantity received:** | 02 Slants | | |
| **Sample packing:** | Sealed Ziploc Pouch | **Condition on receipt:** | Good |
| **Sampling:** | NOT SAMPLED BY EUROFINS | | |

| Analysis | | | *Method* | *Result* | *Unit* |
|---|---|---|---|---|---|
| **DT00E** | **DT** | * External Processing of Orders | NULL | Nhe-/HbL- production negative | /sample |
| **UMDNF** | **DT** | * Bacillus Cereus | Duopath® Cereus Enterotoxins, Merck Duopath Cereus, Immunochromatography | Not Detected | /sample |

**Conclusion :**

No diarrhoe toxin production of the strains (relevant components HbL or Nhe) could be detected.

This report is revised to include Conclusion.

The tests identified by the two letters code DT are subcontracted to our Eurofins Group lab.

**Ms Jyoti Sindhu**

**Manager Lab**

This report supersedes Test Report No. AR-20-IR-020763-02, dated 20/04/2020

*=The parameter with * mark are not covered by our NABL scope.

************** END OF REPORT ***************

The results may not be reproduced except in full, without a written approval of the laboratory. The results relate only to the sample analysed.

ANNEX K

Advanced Enzyme Technologies Ltd.

**Title: Evaluation of antimicrobial activity of Chitosanase enzyme produced by *Bacillus subtilis* (strain CSSC).**

Unique Study Code : BSCS-AET-Q-MICRO-030

Date: 07.11.2014

| | |
|---|---|
| | |
| Study Director | Study Monitor |
| Mr.Animesh Bagchi<br>Advanced Enzyme Technologies Ltd.<br>A 61/62 Malegaon MIDC, Sinnar<br>Nashik-422113 India.<br>E-mail: animesh@advancedenzymes.com | Mr.Santosh Kasliwal<br>Advanced Enzyme Technologies Ltd.<br>A 61/62 Malegaon MIDC, Sinnar<br>Nashik-422113 India.<br>E-mail: santosh@advancedenzymes.com |

Evaluation of antimicrobial activity of a Chitosanase preparation .
Unique Study Code: BSCS-AET-Q-MICRO-030

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*3. Summary*

Chitosanase preparation produced by the strain *Bacillus subtilis* (strain CSSC) is evaluated for the antibacterial activity. This study is carried out on concentrated enzyme preparation.

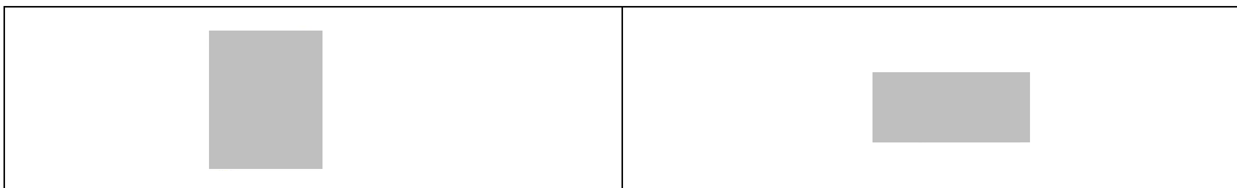
Three batches of Chitosanase were checked for antimicrobial activity as per JECFA (2006) guidelines. Test strains required for the microbiological assay were purchased from American Type Culture Collection (ATCC). Test strains received, revived and checked following ATCC guidelines and bio-chemically characterised using Himedia Laboratories ready-made kits. Purity and viability of ATCC cultures were found to be in accordance with certificate of analysis (ATCC) and biochemical characteristics in compliance with Hi Media Laboratory's interpretation chart .

The results from this study show the absence of antibacterial activity in the three tested batches of Chitosanase preparation.

*4. Quality statement*

This study was carried out in compliance with current quality standards for food additives and following specifications set by Joint FAO/WHO Expert Committee on Food Additives (2006).

Date: 07.11.2014



| Name : Mr.Animesh Bagchi<br>Study Director | Name : Santosh Kasliwal<br>Study Monitor |
|---|---|

*5. Introduction*

The evaluation of the antibacterial agents in food/feed enzyme preparation is important for the safety assessment (EFSA, 2009). The present study describe a test carried out to evaluate presence of antibacterial agent/s in enzyme product.

*6. Study title and Unique Study Code*

Evaluation of antibacterial activity of Chitosanase enzyme produced by *Bacillus subtilis* (strain CSSC).

Unique Study Code: BSCS-AET-Q-MICRO-030

*7. Study objective*

The objective of this study is to evaluate the antibacterial activity in 3 batches of Chitosanase against the selected test microorganisms.

*8. Study location*
Microbiology Lab ,
Advanced Enzyme Technologies Ltd.
Plot No A 61/62,Malegaon MIDC,
Tal Sinnar, Dist- Nasik, Pin - 422113, M. S., India.

*9. Dates of the study*

30/10/2014 to 05/11/2014

*10. Details of test samples*
*10.1.* Chitosanase

Table 1. Details of test samples

| Product name | Manufacturer | Lot no., manufacture & expiry date | Activity U/g |
|---|---|---|---|
| Chitosanase produced from *Bacillus subtilis* (strain CSSC). | Advanced Enzyme Technologies Ltd. | Lot no: 061434<br>Manufacture: June 2014<br>Expiry: May 2016 | 2875 U/g |
| | | Lot no: 101423<br>Manufacture: October 2014<br>Expiry: September 2016 | 2648 U/g |
| | | Lot no: 101424<br>Manufacture: October 2014<br>Expiry: September 2016 | 2986 U/g |

*11. Materials & methods*

*11.1 Assessment of antimicrobial activity in the Chitosanase enzyme*

The antimicrobial activity of Chitosanase produced by *Bacillus subtilis* (strain CSSC) was assessed according to guidelines set by Joint FAO/WHO Expert Committee on Food Additives (2006) as recommended by EFSA (2008 & 2009). The potential antimicrobial activity of Chitosanase was evaluated based on the measurement of inhibition of bacterial growth under specific conditions. The test strains required for the assays were purchased from the American Type Culture Collection (ATCC), USA. The following test micro organisms were used in this evaluation:

- *Staphylococcus aureus* (ATCC 6538); *Escherichia coli* (ATCC 11229); *Bacillus cereus* (ATCC 2); *Bacillus circulans* (ATCC 4516); *Streptococcus pyrogenes* (ATCC 12344) and *Serratia marcescens* (ATCC 14041)

Various nutrient media such as Nutrient Broth/Agar (NB/NA), Trypticase Soya broth/agar (TSB/TSA), Brain Heart Infusion Broth/agar, microbiological assay medium as well as biochemical test kits for the characterisation of test micro organisms were purchased from Hi-media Laboratories Ltd. (Mumbai, India). The required chemicals & reagents were purchased from Merck India Ltd.

The evaluation of antimicrobial activity of enzyme was carried out in the following two steps

1. Revival analysis of ATCC cultures.
2. Standard microbiological assay to evaluate antimicrobial activity in the Chitosanase enzyme preparation

*11.2 Revival & analysis of ATCC cultures*

Each ATCC culture vial was thoroughly checked upon receipt for physical damage and stored at 4-8°C in a refrigerator until revival. Revival of ATCC cultures was done as per ATCC instructions. Revived strains were analysed for purity, cell and colony morphology as per ATCC instructions. All ATCC cultures were preserved in the form of glycerol stocks.

Each ATCC culture was revived by rehydrating lyophilised cell pellet in 0.5 ml of recommended medium (Annexure I). Two hundred (200) µl and 100 µl of rehydrated ATCC culture was inoculated in 100 ml and 5 ml broth media respectively, and incubated at appropriate conditions recommended by ATCC (Annexure I). Purity of each rehydrated ATCC culture was checked by streaking loopful of culture on appropriate media and incubating at respective temperatures recommended by ATCC (Annexure I). Purity of cultures was described in terms of colony characteristics, gram nature and cell morphology of single well isolated colony on respective media.

Glycerol stocks of well grown ATCC cultures were prepared by mixing 75 ml of culture with 25 ml of 60 % glycerol to reach final glycerol concentration up to 15%. One ml aliquots were distributed in previously sterilized and labelled cryo vials and stored at -70$^{0}$C. ATCC culture and 60% glycerol were streaked on Nutrient Agar plate for purity check. Plates were incubated at recommended temperatures (Annexure I).

*11.3 Standard microbiological assay to evaluate antimicrobial activity in the Chitosanase enzyme preparation*

The standard microbiological assay to assess antimicrobial activity in the Chitosanase preparation was carried out in the following three steps,

*11.3.1 Preparation of Trypticase Soya (Soyabean Casein Digest) agar plates*

Fifteen ml of sterile SCDA butts were poured in sterile petri plates to prepare base agar plates and allowed to solidify.

*11.3.2 Growth of test cultures*

Each culture was inoculated in 10 ml of sterile Trypticase Soya (Soyabean Casein Digest) broth. All tubes were incubated at 37$^{O}$C for 24 hrs.

*11.3.3 Disc Preparation*

Homogenous suspension of enzyme (10%) was prepared by dissolving 10 gm of sample in 100 ml of sterile distilled water. Sterile filter paper disks (Filter Papers No. 740-E, 12.7 mm in diameter) were saturated with enzyme by applying 100 µl of 10% solution to the disk surface. Eighteen discs were prepared per batch.

*11.3.4 Microbiological assay*

Each well grown culture (1ml, as described in 11.5.2) was seeded in 9 ml of pre-cooled and sterile SCDA butts and mixed properly. Seeded SCDA was overlaid on previously prepared SCDA agar plates (see 11.5.1) and allowed to solidify. Single disc was placed on each inoculated SCDA plates. Plates were kept in the refrigerator overnight to obtain proper diffusion and then incubated the at 37° for 24 h.

*11.3.5 Observations and interpretations:*

The plates were examined for any inhibition zones around paper discs. A visually clear zone around a disk (total diameter: 16 mm) indicates the presence of antibacterial components in the enzyme preparation. If an enzyme preparation shows obvious antibacterial activity against three (or more) organisms, it is concluded that antimicrobial agents are present.

*12. Results & discussion*

*12.1 Revival & testing of ATCC cultures*

All the test ATCC cultures grew well within 16-18 hours upon revival. All cultures were pure and their colony characteristics, cell morphologies and Gram nature were in accordance with their corresponding certificates of analysis.

*12.2 Microbiological assays to evaluate antimicrobial activity in Chitosanase enzyme preparation*

Clearance zones were not observed for any of the 3 batches of preparation. Results are given in table 2.

Table 2. Observations of clearance zones for 3 batches

| ATCC Culture | Plate No | Batch no 061434 | Batch no 101423 | Batch no 101424 |
|---|---|---|---|---|
| *Staphylococcus aureus* ATCC 6538 | 1 | - | - | - |
| | 2 | - | - | - |
| | 3 | - | - | - |
| *Escherichia coli* ATCC 11229 | 1 | - | - | - |
| | 2 | - | - | - |
| | 3 | - | - | - |
| *Bacillus cereus* ATCC 2 | 1 | - | - | - |
| | 2 | - | - | - |
| | 3 | - | - | - |
| *Bacillus circulans* ATCC 4516 | 1 | - | - | - |
| | 2 | - | - | - |
| | 3 | - | - | - |
| *Streptococcus pyrogenes* ATCC 12344 | 1 | - | - | - |
| | 2 | - | - | - |
| | 3 | - | - | - |
| *Serratia marcescens* ATCC 14041 | 1 | - | - | - |
| | 2 | - | - | - |
| | 3 | - | - | - |

Notes: - = no zone of clearance

*13. Conclusions*

The evaluation of Chitosanase enzyme preparation showed the absence of antimicrobial activity.

*14. References*

EFSA(2009). EFSA Guidance prepared by the Scientific Panel of Food Contact Material, Enzymes, Flavourings and Processing Aids on the Submission of a Dossier on Food Enzymes. *The EFSA Journal* 1305: 1-26.

EFSA(2008). Update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance: Prepared by the panel on additives and products or substances used in animal feed. *The EFSA Journal* 732: 1-15.

FAO/JECFA (2006).Joint FAO/WHO Expert Committee on Food Additives Monographs: Combined compendium of food additive specifications, Volume 4, pp122-123.

*15. Abbreviations*

Table 3. Abbreviations used

| | | | |
|---|---|---|---|
| ATCC | American Type Culture Collection | NB | Nutrient broth |
| BHI | Brain heart infusion | EFSA | European Food Safety Authority |
| SCDA | Soyabean Casein Digest | TSB | Trypton soya broth |
| JECFA | Joint FAO/WHO Expert Committee on Food Additives | TSA | Trypton soya agar |
| µl | Microlitre | FAO | Food and Agriculture Organization |
| ml | Millilitre | WHO | World Health Organisation |
| NA | Nutrient agar | | |

*16. List of Appendices*

*Appendix 1–Growth requirements of ATCC cultures*

| Table 4. Growth requirements of ATCC cultures | | | | | |
|---|---|---|---|---|---|
| Culture name | ATCC nº | Medium used | Make | Catalogue nº | Incubation |
| *Escherichia coli* | 11229 | Nutrient broth or agar | HiMedia | M002 | 37°C |
| *Streptococcus pyrogenes* | 12344 | Brain heart infusion broth or agar | HiMedia | M210 | 37°C |
| *Serratia marcescens* | 14041 | Nutrient broth or Agar | HiMedia | M002 | 26°C |
| *Bacillus cereus* | 2 | Nutrient broth or Agar | HiMedia | M002 | 30°C |
| *Bacillus circulans* | 4516 | Nutrient broth or agar | HiMedia | M002 | 30°C |
| *Staphylococcus aureus* | 6538 | Trypton soya broth or agar | HiMedia | M011 | 37°C |