## Appendix 10: Procedure for the Determination of Inhibitors and Activators

**1. Aim:** To determine the effect of metal ions and EDTA on the enzyme.

**2. Scope:** This method is applicable to the metal salts and EDTA listed below at 1 mM concentration.

**3. Materials:**

3.1. Reagents: Refer to Appendix 4.

3.2. Instruments: Refer to Appendix 5.

**4. Methodology**

4.1. Reagents

4.1.1. Olive Oil Substrate: Refer to Appendix 6.

4.1.2. 0.5 % Sodium taurocholate solution.

4.1.3. 0.02 N Sodium hydroxide solution.

4.1.4. 10 mM Tris-HCl buffer, pH 7.0.

4.1.5. Enzyme/Sample Preparation:

Prepare the dilutions in 10 mM Tris-HCl buffer, pH 7.0 to obtain the readings within range.

4.1.6. Preparation of Metal Salt/EDTA Solutions: Prepare 95 mM solutions of the following metal salts in Milli-Q water: NaCl, KCl, $CoCl_2$, $MgCl_2$, $ZnSO_4$, $CaCl_2$, $CuCl_2$, $MnCl_2$, $HgCl_2$ and EDTA.

4.2. Assay Procedure:

i. Mix 0.3 ml enzyme, 0.6 ml metal salt/EDTA solution and 2.1 ml of distilled water, and incubate at 25°C for 5 minutes.

ii. Mix 11.5 ml of the substrate, 4.5 ml distilled water, 1 ml 0.5% sodium taurocholate solution, equilibrate at 37°C, and adjust the pH to 7.0 using 0.02 N sodium hydroxide.

iii. Add 2 ml of the enzyme-metal ion-mixture, and maintain the pH to 7.0 for 10 minutes at 37°C with 0.02 N sodium hydroxide solution.

iv. At the end of 10 minutes, abruptly bring the pH to 9.0 by adding additional 0.02 N sodium hydroxide solution. Record the volume of sodium hydroxide consumed as $T_{s.}$

v. Analyse the 'Blank' in the same manner, except after equilibration, bring up the pH to 9.0, add the enzyme-metal ion- mixture and again bring the pH back to 9.0. Record the volume of NaOH consumed $T_{b.}$

vi. Set up a 'Control' by adding Milli-Q water instead of the metal salt/EDTA solution and analyze in the same way as mentioned above.

**5. Calculations**: Refer to Appendix 6.

**6. Data Interpretation:**

The enzyme activity without metal ions/EDTA is considered as the 100% activity and based on this the percentage enzyme activity is calculated.

TÜV INDIA
Member of TÜV NORD Group

# Test Report

TUV INDIA PRIVATE LIMITED
TÜV India House,
Survey No. 42, 3/1 & 3/2,
Sus, Tal. Mulshi,
Dist. Pune - 411 021
Tel. : 020 – 67900000 / 01
Email : pune@tuv-nord.com
Website : www.tuvindia.co.in

**Report No : TUV(I)/2766/13-14/0081300765**

**Date : 23 Aug 2013**

Name & Address of Customer : Advanced Enzyme Technologies Ltd.
Plot No A 61/62,
Malegaon MIDC, Sinnar,
Nashik - 422103

Reg No. : 2766/13-14

CA No. : 0081300765

Date of sample receipt : 17 Aug 2013

Date(s) of analysis : 17 Aug 2013 -23 Aug 2013

Sample Drawn by : Customer

| Sl No | Test Name | Result | Unit | Test Method |
|---|---|---|---|---|
| | **Sample Name : Rhizopus Lipase**<br>Batch No.: 0512178<br>Qty.: 1 X 100g | Mfg.: May 2012 | | **CA No : 0081300765** |
| | **Heavy Metals** | | | |
| 1 | Arsenic | <0.25 | mg/kg | Based on AOAC 984.27 & 999.10, 18th edition & by ICP-MS |
| 2 | Cadmium | <0.25 | mg/kg | Based on AOAC 984.27 & 999.10, 18th edition & by ICP-MS |
| 3 | Lead | <0.25 | mg/kg | Based on AOAC 984.27 & 999.10, 18th edition & by ICP-MS |
| 4 | Mercury | <0.025 | mg/kg | Based on AOAC 984.27 & 999.10, 18th edition & by ICP-MS |

TUV India Laboratory Division

Verified by

Shriram Kulkarni
Head - Laboratory Instrumentation

Authorized by

V. K. Gupta
General Manager - Laboratory Operations

-- End of Report --

1. Test Results are based on & related only to the particular sample(s) tested.
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5. This Report, in full or in part, shall not be used to make any misleading claims or for any legal purposes.

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# Test Report

**TUV INDIA PRIVATE LIMITED**
TÜV India House,
Survey No. 42, 3/1 & 3/2,
Sus, Tal. Mulshi,
Dist. Pune - 411 021
Tel. : 020 – 67900000 / 01
Email : pune@tuv-nord.com
Website : www.tuvindia.co.in

**Report No : TUV(I)/2766/13-14/0081300766**

**Date : 23 Aug 2013**

Name & Address of Customer : Advanced Enzyme Technologies Ltd.
Plot No A 61/62,
Malegaon MIDC, Sinnar,
Nashik - 422103

Reg No. : 2766/13-14

CA No. : 0081300766

Date of sample receipt : 17 Aug 2013

Date(s) of analysis : 17 Aug 2013 -23 Aug 2013

Sample Drawn by : Customer

| Sl No | Test Name | Result | Unit | Test Method |
|---|---|---|---|---|
| | **Sample Name : Rhizopus Lipase**<br>Batch No.: 0413134<br>Qty.: 1 X 100g | Mfg.: Apr 2013 | | **CA No : 0081300766** |
| | **Heavy Metals** | | | |
| 1 | Arsenic | <0.25 | mg/kg | Based on AOAC 984.27 & 999.10, 18th edition & by ICP-MS |
| 2 | Cadmium | <0.25 | mg/kg | Based on AOAC 984.27 & 999.10, 18th edition & by ICP-MS |
| 3 | Lead | <0.25 | mg/kg | Based on AOAC 984.27 & 999.10, 18th edition & by ICP-MS |
| 4 | Mercury | <0.025 | mg/kg | Based on AOAC 984.27 & 999.10, 18th edition & by ICP-MS |

TUV India Laboratory Division

Verified by
(b) (6)
Shriram Kulkarni
Head - Laboratory Instrumentation

Authorized by
(b) (6)
V. K.
General Manager - L erations

-- End of Report --

1. Test Results are based on & related only to the particular sample(s) tested.
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5. This Report, in full or in part, shall not be used to make any misleading claims or for any legal purposes.

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TÜV NORD GROUP

**TUV INDIA PRIVATE LIMITED**
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CIN : U74140MH1989PTC052930
Tel. : 020 – 67900000 / 01
Email : pune@tuv-nord.com
Website : www.tuv-nord.com/in

# Test Report

**Report No : TUV(I)/2331/14-15/0071400444**

**Date : 14 Jul 2014**

Name & Address of Customer : Advanced Enzymes Technologies Ltd
Plot No A 61/62,
Malegaon MIDC, Sinnar,
Nashik - 422103

Reg No. : 2331/14-15

CA No. : 0071400444

Date of sample receipt : 09 Jul 2014

Date(s) of analysis : 09 Jul 2014 -14 Jul 2014

Sample Drawn by : Customer

| Sl No | Test Name | Result | Unit | Test Method |
|---|---|---|---|---|
| | **Sample Name : Rhizopus Lipase**<br>Batch No: 011423 | | | **CA No : 0071400444** |
| | **Heavy Metals** | | | |
| 1 | Arsenic | <0.1 | mg/kg | Based on AOAC 984.27 & 999.10 18th edition b y ICP-MS |
| 2 | Cadmium | <0.1 | mg/kg | Based on AOAC 984.27 & 999.10,18th edition b y ICP-MS |
| 3 | Lead | <0.1 | mg/kg | Based on AOAC 984.27 & 999.10,18th edition b y ICP-MS |
| 4 | Mercury | <0.025 | mg/kg | Based on AOAC 984.27 & 999.10,18th edition b y ICP-MS |

Verified by
(b) (6)
Lalitkumar Thakur
Section Incharge – Instrumentation

Authorized by
(b) (6)
Rehana Sheikh
Sr. Laboratory Analyst

-- End of Report --

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5. This Report, in full or in part, shall not be used to make any misleading claims or for any legal purposes.

# ANNEX E1

TNO triskelion bv

## Analysis report

Utrechtseweg 48
3704 HE Zeist
P.O. Box 844
3700 AV Zeist
The Netherlands

www.triskelion.nl

T +31 88 866 28 00
info@tno.triskelion.nl

| | |
|---|---|
| **TNO reference (Submission ID)** | 110088548 |
| **Date received** | 17 January 2014 |
| **Company name** | Advanced Enzym Technologies Ltd. |
| **Sample description** | Rhizopus Lipase |
| **TNO sample ID** | 20121-0788 |
| **Customer sample ID** | batch: 011423 |

**Report date**
6 February 2014

**Report status**
Final report

**Report number**
20121-00101

**Project number**
093.20621/03.19.01

**Your reference**
January 14, 2014

**Direct dialing**
+31 888 66 17 67

| Analyte | Result | Unit |
|---|---|---|
| aflatoxin B1 | < 1 | µg/kg |
| aflatoxin B2 | < 1 | µg/kg |
| aflatoxin G1 | < 1 | µg/kg |
| aflatoxin G2 | < 1 | µg/kg |
| aflatoxin M1 | < 1 | µg/kg |
| ochratoxin A | < 1 | µg/kg |
| fumonisin B1 | <100 | µg/kg |
| zearalenone | < 5 | µg/kg |
| deoxynivalenol (DON) | < 25 | µg/kg |
| T2-toxin | < 10 | µg/kg |
| HT2-toxin | < 50 | µg/kg |
| ergocornine | <100 | µg/kg |
| ergocristine | <100 | µg/kg |
| ergocryptine | <100 | µg/kg |
| ergometrine | <100 | µg/kg |
| ergosine | <100 | µg/kg |
| ergotamine | <100 | µg/kg |



### Method(s) used

| Analysis method | SOP-code | Technique |
|---|---|---|
| (1) mycotoxins multi method | TRIS/MYC/024 | LC-MS/MS |

Samples were extracted with acetonitrile/water for 1.5 hours. The solutions were centrifuged and diluted. The extracts were analyzed for the possible presence of mycotoxins by means of high performance liquid chromatography in combination with Quadruple MS-MS ESI (LC-MS).

Approval
(b) (6)

A. Schouten
Study director

TNO triskelion bv

# Analysis report

Utrechtseweg 48
3704 HE Zeist
P.O. Box 844
3700 AV Zeist
The Netherlands

www.triskelion.nl

T +31 88 866 28 00
info@tno.triskelion.nl

| | |
|---|---|
| **TNO reference (Submission ID)** | 110088548 |
| **Date received** | 17 January 2014 |
| **Company name** | Advanced Enzym Technologies Ltd. |
| **Sample description** | Rhizopus Lipase |
| **TNO sample ID** | 20121-0789 |
| **Customer sample ID** | batch: 0512178 |

**Report date**
6 February 2014

**Report status**
Final report

**Report number**
20121-00101

**Project number**
093.20621/03.19.01

**Your reference**
January 14, 2014

**Direct dialing**
+31 888 66 17 67

| Analyte | Result | Unit |
|---|---|---|
| aflatoxin B1 | < 1 | µg/kg |
| aflatoxin B2 | < 1 | µg/kg |
| aflatoxin G1 | < 1 | µg/kg |
| aflatoxin G2 | < 1 | µg/kg |
| aflatoxin M1 | < 1 | µg/kg |
| ochratoxin A | < 1 | µg/kg |
| fumonisin B1 | <100 | µg/kg |
| zearalenone | < 5 | µg/kg |
| deoxynivalenol (DON) | < 25 | µg/kg |
| T2-toxin | < 10 | µg/kg |
| HT2-toxin | < 50 | µg/kg |
| ergocornine | <100 | µg/kg |
| ergocristine | <100 | µg/kg |
| ergocryptine | <100 | µg/kg |
| ergometrine | <100 | µg/kg |
| ergosine | <100 | µg/kg |
| ergotamine | <100 | µg/kg |



## Method(s) used

| Analysis method | SOP-code | Technique |
|---|---|---|
| (1) mycotoxins multi method | TRIS/MYC/024 | LC-MS/MS |

Samples were extracted with acetonitrile/water for 1.5 hours. The solutions were centrifuged and diluted. The extracts were analyzed for the possible presence of mycotoxins by means of high performance liquid chromatography in combination with Quadruple MS-MS ESI (LC-MS).

Approval
(b) (6)

A. Schouten
Study director

TNO triskelion bv

# Analysis report

Utrechtseweg 48
3704 HE Zeist
P.O. Box 844
3700 AV Zeist
The Netherlands

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T +31 88 866 28 00
info@tno.triskelion.nl

| | |
|---|---|
| **TNO reference (Submission ID)** | 110088548 |
| **Date received** | 17 January 2014 |
| **Company name** | Advanced Enzym Technologies Ltd. |
| **Sample description** | Rhizopus Lipase |
| **TNO sample ID** | 20121-0790 |
| **Customer sample ID** | batch: 0413134 |

**Report date**
6 February 2014

**Report status**
Final report

**Report number**
20121-00101

**Project number**
093.20621/03.19.01

**Your reference**
January 14, 2014

**Direct dialing**
+31 888 66 17 67

| Analyte | Result | Unit |
|---|---|---|
| aflatoxin B1 | < 1 | µg/kg |
| aflatoxin B2 | < 1 | µg/kg |
| aflatoxin G1 | < 1 | µg/kg |
| aflatoxin G2 | < 1 | µg/kg |
| aflatoxin M1 | < 1 | µg/kg |
| ochratoxin A | < 1 | µg/kg |
| fumonisin B1 | <100 | µg/kg |
| zearalenone | < 5 | µg/kg |
| deoxynivalenol (DON) | < 25 | µg/kg |
| T2-toxin | < 10 | µg/kg |
| HT2-toxin | < 50 | µg/kg |
| ergocornine | <100 | µg/kg |
| ergocristine | <100 | µg/kg |
| ergocryptine | <100 | µg/kg |
| ergometrine | <100 | µg/kg |
| ergosine | <100 | µg/kg |
| ergotamine | <100 | µg/kg |



## Method(s) used

| Analysis method | SOP-code | Technique |
|---|---|---|
| (1) mycotoxins multi method | TRIS/MYC/024 | LC-MS/MS |

Samples were extracted with acetonitrile/water for 1.5 hours. The solutions were centrifuged and diluted. The extracts were analyzed for the possible presence of mycotoxins by means of high performance liquid chromatography in combination with Quadruple MS-MS ESI (LC-MS).

Approval
(b) (6)

A. Schouten
Study director

**ANNEX F**

# Hazard Analysis and Critical Control Points (HACCP) of *Rhizopus oryzae* lipase produced by genetically modified *Aspergillus niger* agg. (strain FL100SC)

| | |
|---|---|
| (b) (6) | (b) (6) |
| Prepared By | Checked and Approved |
| Mr. Chandrakant Wadnere<br>Asst. Manager Quality Assurance | Mr. Animesh Bagchi<br>Manager, Quality Assurance |

## TABLE OF CONTENTS

## 1 Purpose

HACCP is a systematic approach which identifies specific hazards and provides measures for their control to ensure the safety of the product. HACCP is a tool to assess hazards and establish control systems that focus on prevention rather than relying on end product testing.

The HACCP application must be reviewed and necessary changes made when any modification is made in the process, equipment and product.

## 2 Hazard Classification

A hazard can be described as a contaminant which can have adverse consequences for the health of humans and animals. Three types of hazard are distinguished:

***Chemical hazards (C)***
Are undesirable chemical elements which may make the product unsafe? They may be present in the raw materials or may contaminate the product during the production process. Examples are residues of heavy metals, mycotoxins, etc.

***Microbiological hazards (M)***
Relate to the presence of undesirable micro-organisms. The microorganisms can, as a result of their (natural) presence, contamination or development, result in a product being unsafe. A distinction is made among vegetative micro-organisms, toxigenic (toxin forming) micro-organisms and spore forming micro-organisms. Examples are E. coli, Salmonella, Pseudomonas, Enterobacteriaceae and “Moulds and Yeasts” (the latter group as indicator organisms).

***Hazards (P)***

Physical hazards are foreign bodies such as glass, plastic, metal components, stones, etc., which may be present in the raw materials or contaminate the product. This makes the product unsafe.

## 3 CCP decision tree

Each identified hazard must be assessed by using the CCP (Critical Control Point) decision tree. The CCP decision tree is used to determine whether a hazard must be controlled using a specific control measure (CCP), a general measure POA (Point Of Attention) or another periodic measure.

## 4 Risk assessment

Risk assessment is a method by which risk class can be defined. Risk is determined by two elements, ‘severity’ and ‘probability’ of a potential hazard. The assurance for each risk class is characterized by (a combination of) control measures.

| Severity | Probability of occurrence | | | |
|---|---|---|---|---|
| Great | 2 | 3 | 4 | 4 |
| Medium | 1 | 2 | 3 | 4 |
| Small | 0 | 1 | 2 | 3 |
| | < Small | Small | Medium | Great |

***Severity*** is the consequence for the consumer when exposed to the hazard. Severity is divided into three levels:

Great (G): Fatal consequences, serious illness, and irrecoverable injuries either immediately or in the longer term.

Medium (M): Substantial injuries and/or illness, occurring either immediately or in the longer term.

Small (S): minor injuries and/or illness, not or hardly occurring or only in extremely high doses for a long period of time.

The probability is the frequency that the hazard will occur in the end product. The probability is based on measurements, observations or expectations in a company specific situation and it is divided into four levels:

<Small (<S): seems to be impossible

Small (S): practically not probable

Medium (M): might occur, it has been known to occur

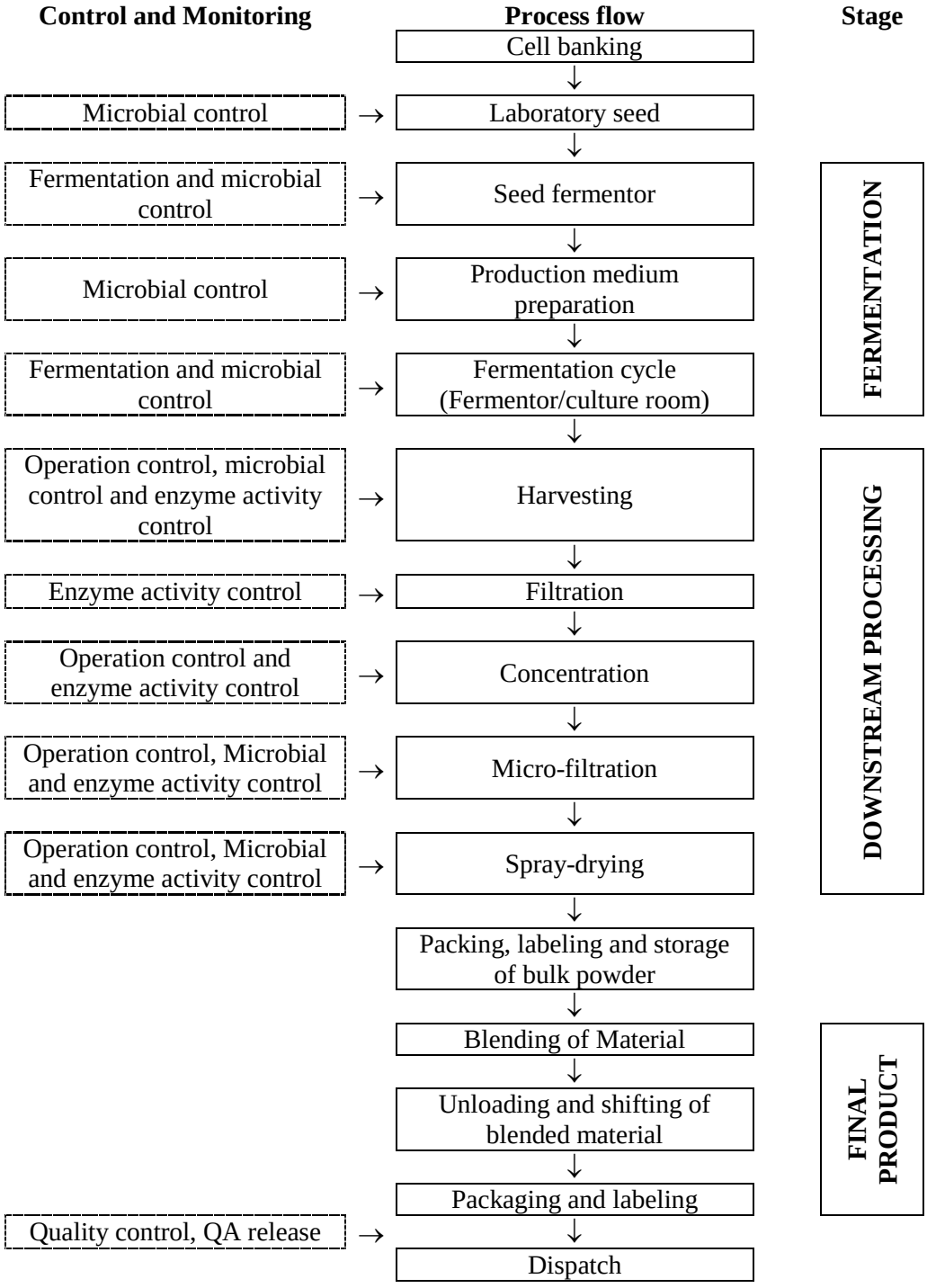
Great (G): occurs repeatedly

## 5 Risk class and Control measures

| Risk Class | Control measures |
|---|---|
| 0 | No measures necessary |
| 1 | No measures necessary |
| 2 | Measuring and monitoring of the process parameters to ensure that they are within the specified range. These measures must be reviewed periodically against the information available |
| 3 | General control measures are often called POA's (Points of Attention) and includes action based on the type of activity for which these are mentioned. |
| 4 | Specific control measures are specially developed and used to control the risk. |

## 6 Process flow diagram

A detailed process flow diagram with control and monitoring points is given below:



# 7 Risk Analysis Table(s)

## *7.1 Cell bank and laboratory seed*

| No. | Process step | Hazard description | Category | Probability | Severity | Risk | Type of measures | CCP / POA | Motivation |
|---|---|---|---|---|---|---|---|---|---|
| 7.1.1 | Growth of production strain in laboratory seed | Contamination with other organism | M | M | S | 2 | (i) Restricted entrance, (ii) Hygiene measurements, (iii) Use of HVAC, and (iv) Laminar work flow stations | - | Without very strict hygiene it is possible that the seed culture is contaminated. There is no risk to final product as the contaminated seed is discarded. |

## *7.2 Seed fermentor*

| No. | Process step | Hazard description | Category | Probability | Severity | Risk | Type of measures | CCP / POA | Motivation |
|---|---|---|---|---|---|---|---|---|---|
| 7.2.1 | Propagation of culture to increase biomass | Incorrect material used in the seed fermenter. | C | S | M | 2 | (i) Receipt and verification procedures are in place, (ii) Issue and receipt of material done by two different individual, (iii) Batch wise issue and receipt of material which is recorded in batch card. | - | As the media ingredients possessnutritional property, the growth of the production organism will not be affected. However with strict controls, issue of incorrect material is avoided. There is no risk to the final product as the nutrients are consumed by the production organism. |
| 7.2.2 | | Contamination with other organism | M | M | S | 2 | (i) Controlled procedure for culture inoculation from lab seed, (ii) Hygiene measurements, (iii) Sterilizable SS Vessels designed for aseptic operations. | - | Without very strict hygiene it is possible that the seed culture is contaminated. There is no risk to the final product as the contaminated seed is discarded. |

## *7.3 Production medium preparation*

| No. | Process step | Hazard description | Category | Probability | Severity | Risk | Type of measures | CCP / POA | Motivation |
|---|---|---|---|---|---|---|---|---|---|
| 7.3.1 | Loading of raw material in fermenter, sterilization and cooling. | Incorrect material used in the fermentation process. | C | S | M | 2 | (i) Receipt and verification, procedures are in place, (ii) Issue and receipt of material done by two different individuals., (iii) Batch wise issue and receipt of material which is recorded in batch card. | - | As the media ingredients possess nutritional property, the growth of the production organism will not be affected. However with strict controls, issue of incorrect material is avoided. There is no risk to final product as the nutrients are consumed by the production organism. |

## *7.4 Fermentation cycle*

| No. | Process step | Hazard description | Category | Probability | Severity | Risk | Type of measures | CCP / POA | Motivation |
|---|---|---|---|---|---|---|---|---|---|
| 7.4.1 | Production of enzymes | Contamination with other organism | M | M | S | 2 | (i) Closed production system designed for aseptic operations, (ii) Culture transfer from seed vessel through closed loop, (iii) Hygienic condition maintenance. | - | Without very strict hygiene it is possible that the fermenter is contaminated. There is no risk to final product as the batch is discarded. |

## 7.5 Filtration

| No. | Process step | Hazard description | Category | Probability | Severity | Risk | Type of measures | CCP / POA | Motivation |
|---|---|---|---|---|---|---|---|---|---|
| 7.5.1 | It is removal of unused media components and cell debris of the production organism from the enzyme which is in liquid phase | Contamination with other organism | M | M | S | 2 | (i) Closed production system, (ii) Hygienic condition maintenance. | - | Without very strict hygiene it is possible that the filtrate is contaminated. There is no risk to final product safety due to several controls in the production in a later stage. (Microfiltration step, viable count / pathogen testing). |
| 7.5.2 | | Product that is not sufficient filtered. (Clarity of filtrate not obtained) | P | M | S | 2 | SOP for repeating the filtration till it is sufficient (Clear solution is obtained). | - | Due to the SOP for repeating the filtration the probability is small. But even when it happens the severity will be small due to the fact that re filtration is carried out for efficient processing of further steps. |

## 7.6 Concentration

| No. | Process step | Hazard description | Category | Probability | Severity | Risk | Type of measures | CCP / POA | Motivation |
|---|---|---|---|---|---|---|---|---|---|
| 7.6.1 | It is increasing the enzyme concentration by removing water and other impurities. | Contamination with other organism | M | M | S | 2 | (i) Closed production system, (ii) Hygienic condition maintenance. | - | Without very strict hygiene it is possible that the concentrated solution is contaminated. There is no risk to final product safety due to several controls in the production in a later stage (Microfiltration step, viable count / pathogen testing). |

## *7.7 Microfiltration*

| No. | Process step | Hazard description | Category | Probability | Severity | Risk | Type of measures | CCP / POA | Motivation |
|---|---|---|---|---|---|---|---|---|---|
| 7.7.1 | It is carried out to reduce the bio burden in the enzyme solution. | Contamination with other organism | M | M | S | 2 | (i) Closed production system, (ii) Hygienic condition maintenance. | - | Without very strict hygiene it is possible that the micro filtered solution is contaminated. There is no risk to final product safety due to strict analytical control (viable count / pathogen testing). |

## *7.8 Spray-drying*

| No. | Process step | Hazard description | Category | Probability | Severity | Risk | Type of measures | CCP / POA | Motivation |
|---|---|---|---|---|---|---|---|---|---|
| 7.8.1 | It is conversion of enzyme liquid to powder form | Moisture content of the product is too high and by that more sensitive for microbial growth. | F/M | M | S | 2 | SOP | -- | When air temperature is not correct or time is too short it is possible that the moisture stay too high. The material with high moisture is not used in the production of finished product. |
| 7.8.2 | | Product is damaged through air temperature above maximum. | F | M | S | 2 | SOP | -- | When the air temperature is too high the product will be damaged. Enzymes will loss activity. The material with low activity is not used in the production of finished product. |

## *7.9 Blending*

| No. | Process step | Hazard description | Category | Probability | Severity | Risk | Type of measures | CCP / POA | Motivation |
|---|---|---|---|---|---|---|---|---|---|
| 7.9.1 | Blending of material | Presence of chemical contaminants in excess of the norms set (heavy metals and Mycotoxins) in the carriers or residues of earlier productions | C /P | M | M | 3 | (i) Purchase procedure, (ii) Receipt and verification procedures, (iii) Periodic analyses (Once in 6 months), (iv) SOP for cleaning | POA 1 | Although both carriers are bought with Pharmaceutical / Food grade qualities it is not totally impossible (but very unlikely) that these contaminants are present. However unlikely, due to the inclusion of the final product, the final product can get contaminated with undesirable substances. |
| 7.9.2 | | Mixture is not homogenous | C | S | S | 1 | (i) Analysis of final blend prior to sale, (ii) Validated blending process | - | Due to insufficient mixing because of deviation in the mixing time it is possible that the mixture is not homogenous. The final product is analyzed prior to dispatch and hence there is control in the final product quality. |

## *7.10 Packaging, labeling and storage*

| No. | Process step | Hazard description | Category | Probability | Severity | Risk | Type of measures | CCP / POA | Motivation |
|---|---|---|---|---|---|---|---|---|---|
| 7.10.1 | Packaging, labeling and storage of final product | Wrong use of the product because of a mistake in labels / package | C | S | S | 1 | (i) SOP for packing, (ii) Batch card of product, (iii) Final analysis | - | It is a remote possibility that wrong labels are used. The final check done by the QA will take care of this is deviation if any. |
| 7.10.2 | | Wrong use of the product because of a mistake in package weight | C | S | S | 1 | (i) SOP for packing, (ii) Batch card of product, (iii) Final analysis | - | It is a remote possibility that the package is filled with the wrong weight. The quality of the final product is not affected. |
| 7.10.3 | | Growth of microorganisms through long-term storage. | M | S | M | 2 | (i) Periodic analyses, (ii) SOP for cleaning and hygienic storage | - | Very unlikely as the final product is prepared based on sales forecast. |
| 7.10.4 | | Reduces activity through long-term storage | C | M | S | 2 | (i) Periodic analyses | - | Very unlikely as the final product is prepared based on sales forecast. |

## *7.11 Dispatch*

| No. | Process step | Hazard description | Category | Probability | Severity | Risk | Type of measures | CCP / POA | Motivation |
|---|---|---|---|---|---|---|---|---|---|
| 7.11.1 | Loading | Wrong use of the product because of a mistake in product loaded / unloaded | C | M | S | 2 | SOP for delivering. | - | It is possible that the wrong product is loaded. The material will not be accepted at the customers end and will be returned. |

## 8 Summary of POAs

*Summary of all POAs and the monitoring:*

| Sr. No. | POA | Person Responsible | Monitoring | Corrective actions | By | Documentation |
|---|---|---|---|---|---|---|
| 1 | Periodic analysis on heavy metals and mycotoxins in the carriers | Manager QA | Analyzing | Supplier and ingredient rejected. | MR | Analytical report |

## 9 Summary of CCP

*Summary of all CCP's and the monitoring:*

No CCP Found.

**ANNEX G**

# SAFETY CONSIDERATIONS FOR BIOTECHNOLOGY 1992

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

**ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT**

Pursuant to Article I of the Convention signed in Paris on 14th December 1960, and which came into force on 30th September 1961, the Organisation for Economic Co-operation and Development (OECD) shall promote policies designed:

- to achieve the highest sustainable economic growth and employment and a rising standard of living in Member countries, while maintaining financial stability, and thus to contribute to the development of the world economy;
- to contribute to sound economic expansion in Member as well as non-member countries in the process of economic development; and
- to contribute to the expansion of world trade on a multilateral, non-discriminatory basis in accordance with international obligations.

Theoriginal Member countries of the OECD are Austria, Belgium, Canada, Denmark,France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, the Netherlands, Norway, Portugal, Spain, Sweden, Switzerland, Turkey, the United Kingdom and the United States. The following countries became Members subsequently through accession at the dates indicated hereafter: Japan (28th April 1964), Finland (28th January 1969), Australia (7th June 1971) and New Zealand (29th May 1973). The Commission of the European Communities takes part in the work of the OECD (Article 13 of the OECD Convention). Yugoslavia has a special status at OECD (agreement of 28th October 1961).

Publié en franqais sous le titre :

CONSIDÉRATIONS DE SÉCURITÉ
RELATIVES A LA BIOTECHNOLOGIE - 1992

## FOREWORD

This report is a follow-up to the 1986 publication *Recombinant DNA Safety Considerations* which set out the first international safety guidelines for biotechnology applications to industry, to agriculture and to the environment. A Recommendation of the OECD Council adopted the conclusions and recommendations made in the report and instructed the Committee for Scientific and Technological Policy to review, in consultation with other interested Committees of the Organisation, the experience and action of Member countries in connection with the principles contained therein.

Following the Council Recommendation and instructions, and in response to the unanimous interest expressed by Member countries, the Committee for Scientific and Technological Policy decided at its 46th session, on 10-11 February 1987, to continue to keep safety issues under review and mandated its subsidiary body, the Group of National Experts on Safety in Biotechnology, to carry out a follow-up programme in co-operation with the Environment Committee.

This report deals with two priority issues of this programme which are connected with the important development of biotechnology industrial production and field experiments in Member countries. Namely, it elaborates the initial scientific criteria set forth in 1986 for the safe development, under "Good Industrial Large-Scale Practice" (GILSP), of fermentation-derived biotechnology products and defines "Good Developmental Principles" (GDP) for the design of safe small-scale field research with plants and micro-organisms with newly introduced traits.

On 28 November 1991 the OECD Council agreed to derestrict this report.

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## PREFACE

The issue of safety in biotechnology has been a priority concern of the Committee for Scientific and Technological Policy for a number of years.

In 1983, this committee created a Group of National Experts to consider safety in the use of r-DNA organisms in industry, agriculture and the environment. As a result, general guidelines were published in 1986 in a report entitled *Recombinant DNA Safety Considerations*.

A follow-up programme to that report was started in 1988 and undertaken by the Directorate for Science, Technology and Industry in co-operation with the Environment Directorate of the OECD. The main task of the Group of National Experts was to update and further develop the safety considerations set out in the 1986 report.

This report sets out general principles and criteria for safe large-scale industrial production and small-scale experimental field research in biotechnology, two areas to which the Group of National Experts accorded priority attention in its Mandate.

The report consists of two parts :

- Part One further develops the Good Industrial Large-scale Practice criteria and reviews the fundamental principles, identified in the 1986 report, for the handling of low-risk r-DNA organisms in industrial production.

- Part Two provides guidance on the design of low or negligible risk (small-scale) field research with genetically modified plants and micro-organisms. It introduces general principles for such research, or «Good Developmental Principles» (GDP) applicable to the continuum of testing from laboratory to production release. This continuum is represented diagramatically in a number of stages (Stage 1, Stage 2, Stage 3) on page 20. Although, in general, each stage may be revisited several times, as indicated in the diagram, it was recognised that in some situations satisfactory results may be obtained in a single stage. Therefore, it should be understood that in some cases, and in the light of current experience and adequate knowledge, it may not be necessary to proceed in a progressive step-wise fashion from Stage I to Stage 3.

The scientific principles presented in this report should facilitate the process, started in 1986, of developing consensus on the scientific basis for safe use of biotechnology.

Whilst the Group of National Experts continues to review other issues included in their Mandate, among which biotechnology applied research and food safety, there was agreement that this report should be published without delay to provide Member countries with timely guidance for their growing number of industrial applications and experimental field research.

*Part One*

# ELABORATION OF CRITERIA AND PRINCIPLES FOR GOOD INDUSTRIAL LARGE-SCALE PRACTICE (GILSP)

## BACKGROUND

The OECD report, *Recombinant DNA Safety Considerations,* published in 1986, set out a concept called "Good Industrial Large-Scale Practice (GILSP)" applicable to intrinsically low-risk r-DNA organisms used in industrial production. The concept encompassed certain criteria which an r-DNA organism must meet in order to be given GILSP status. It stated that r-DNA GILSP organisms can be handled, on a large scale, under the same conditions of minimal controls and containment procedures as would be used for the host strains. The key principle for GILSP is that the r-DNA organism should be as safe as the low-risk organism from which it is derived.

An internal survey carried out in 1988 in OECD countries on the use of the GILSP concept or its underlying principles showed that it had been adopted in national guidelines in a number of countries and was being considered for implementation in others. Furthermore, in some countries significant numbers of r-DNA micro-organisms from a limited range of species as well as some r-DNA cell cultures had been assigned GILSP status.

The survey established that:

- there are now a number of examples worldwide of GILSP micro-organisms;
- there appears to be some variation in the extent to which the concept has been taken up in practice;
- there is a general need for a better understanding of the way the concept can be applied and, in particular, for further elaboration of the GILSP criteria.

Part One develops further the criteria and principles for Good Industrial Large-Scale Practice (GILSP) identified in the 1986 report. It is based on current knowledge and experience with use of GILSP organisms. It takes into account the findings of the survey and draws, in part, upon an analysis of 25 examples submitted by Member countries which illustrate application of the GILSP criteria to specific cases. As part of this GILSP update the "Fundamental Principles of Good Occupational Safety and Hygiene", originally given in the 1986 report, have also been further elaborated and entitled "Fundamental Principles of Good Occupational and Environmental Safety".

The material presented in Part One is intended to assist Member countries to identify low-risk organisms that meet the GILSP criteria and to select appropriate practices consistent with GILSP principles. It is anticipated that increasing knowledge and experience with the various applications of the GILSP concept and its underlying principles will allow continued evolution of these safety criteria. Existing information demonstrates that a wide range of organisms are grown safely on a large scale with low risk. Low-risk organisms that do not qualify under the criteria for GILSP may be designated for handling under the same conditions, based on considerations set out in Section I (p. 8).

## I. GENERAL CONSIDERATIONS

An important general point made in the 1986 OECD report is that hazards associated with r-DNA organisms can be assessed and managed like those associated with any other organisms. It is expected that the vast majority of r-DNA organisms to be used in industrial large-scale production can be handled using GILSP.

Irrespective of' the intrinsic safety of the organisms concerned, zero risk is not realistic even for GILSP organisms.

Central to the concept of GILSP are:

- the assessment of' the recombinant organism according to identified criteria to determine that it is as safe as the low-risk host organism;
- the identification and adoption of practices ensuring the safety of the operation.

r-DNA organisms which meet the GILSP criteria and are therefore of low-risk can thus be handled under conditions already found to be appropriate for the relevant hosts.

GILSP therefore lies within the framework of existing safety practices and provides an equivalent to established national and international definitions of the lowest risk category of organisms. It should be emphasized that in the conception and especially in the application of GILSP to date there has been great flexibility as to how the criteria described in Appendix F of the 1986 report are met in individual cases.

GILSP applies to organisms considered to be of low-risk and classified in the lowest risk class. In order to ensure that, for each individual case, an r-DNA organism merits the designation of GILSP, the criteria elaborated in Section II (pp. 10-13) must be taken into consideration in an integrated way. Two clear examples of other classes of organisms that warrant the GILSP designation, provided they are non-pathogenic and without adverse consequences for the environment, are:

i. those constructed entirely from a single prokaryotic host (including its indigenous plasmids and viruses) or from a single eukaryotic host (including its chloroplasts, mitochondria or plasmids -- but excluding viruses); and

ii. those consisting entirely of DNA segments from different species that exchange DNA by known physiological processes.

Organisms that do not meet all the criteria for GILSP are not GILSP organisms. However, after the case-by-case evaluation, they may be found to be of low risk. In such circumstances, these organisms may be handled using GILSP. Care must be taken, when extrapolating GILSP to other organisms, to evaluate whether specific practices in addition to GILSP are required to mitigate a specific concern.

Organisms which can be handled on a large scale under conditions of mininial controls and containment procedures will be:

- those meeting the criteria of Section II (pp. 10-13);

- those other classes of organisms described under points *i)* and *ii)* of the, abovc paragraph;
- other organisms not meeting either of these sets of criteria but which have been demonstrated to be of low-risk, as described above.

When handling GILSP and other low-risk organisms, established principles of good occupational and environmental safety as described in Section III (p. 14) must be followed.

## II. ELABORATION OF CRITERIA FOR R-DNA GILSP (GOOD INDUSTRIAL LARGE-SCALE PRACTICE) MICRO-ORGANISMS AND CELL CULTURES

The criteria outlined below are relevant to micro-organisms and are equally appropriate for cell cultures. It is important that all of the criteria be considered in relation to one another in evaluating the GILSP status of an organism.

### Host[1]

*Non-pathogenic*

The identity of the host must be established and the taxonomy well understood. The host must be evaluated to determine that it is not pathogenic. The host should not appear in national or other recognised lists of human pathogens. Member countries may have additional listings of plant and animal pathogens which may be a useful source of information in assessing the potential of the host to behave as a pathogen. In cases where uncertainty remains for the potential pathogenicity of an organism or an attenuated strain, further data must be developed to confirm its safety and hence its suitability for handling under GILSP conditions. In addition, some organisms not found in pathogen lists may produce toxic substances in amounts which require further evaluation[2].

Examples of hosts that are currently used in GILSP practice are listed below. It should be noted that, in some instances, entire species may qualify for GELSP host status, whereas in other cases only some strains or types may be so designated:

*Saccharomyces cerevisiae*
*Escherichia coli* K-12
*Bacillus subtilis*
CHO (Chinese Hamster Ovary) cells
*Aspergillus oryzae*

*No adventitious agents*

This is mainly relevant to cell cultures where harmful micro-organisms, in particular harmful viruses and mycoplasma, should not be present at detectable levels. Bacterial cultures should not contain unwanted phages.

*Extended history of safe use*

There should be adequate and documented experience of the safe use of the host organism, i.e. without harm to humans or to the environment. Historical and other data on the host, its progenitors or closely related strains may be appropriate for evaluation.

Such evidence may be obtained from applications such as food, enzyme and antibiotic production including from discharge practices used in such applications. Laboratory use and/or pilot scale fermentations under conditions of minimal containment could also provide useful data.

*Built-in environmental limitations permitting optimal growth in industrial setting but limited survival without adverse consequences in the environment*

The possibility of adverse effects can be reduced by restrictions on the organism's ability to multiply, disseminate or survive. This can be achieved by using built-in stable biological limitations which, without interfering with growth in the bio-reactor, dintinish survivability and prevent adverse consequences in the environment.

Examples of organisms with biological limitations include: auxotrophic strains, asporogenic strains, strains with built-in sensitivity to environmental factors, such as UV light, etc.

**Vector/Insert**

*Well characterised and free from known harmful sequences*

*Vector:* For the vector to be well-characterised, the function of the genetic material on the vector should be known.

Vectors can be characterised by a combination of reference to the literature, National Institute of Health (NIH) and/or other listings and a knowledge of the derivation and construction of the vector and subsequent experimental confirmation of the construct.

The characterisation should ensure that the vector is free from sequences that result in a phenotype harmful to humans or the environment; for example, through production of substances which can have harmful effects, such as toxins or factors known to be involved in pathogenicity and/or colonisation.

*Insert:* The source and the function of the DNA that is being inserted and its position on the vector should be known. Experience has shown that in many cases, this means the nucleotide sequence of the inserted DNA is known. This would include knowledge of whether more than one function is encoded in the sequence of the insert. In addition, the insert should not result in a phenotype harmful to humans or the environment as exemplified in the above paragraph.

*Limited in size as much as possible to the DNA required to perform the intended. function; should not increase the stability of the construct in the environment (unless that is a requirement of the intended function)*

The vector/insert should be limited in size as much as possible to the genetic sequences required to perform the intended functions. This decreases the probability of introduction and expression of cryptic functions, or the acquisition of unwanted traits.

In some cases, the vector or the insert may affect the stability of the construct in the environment. For example, introduction of resistance genes may affect the ability of the recipient to survive in the environment (see below).

*Should be poorly mobilisable*

One consideration arising from the use of vectors to introduce an insert is the rate at which the vector/insert could subsequently be transferred from the original recipient. For example, the rate of exchange of plasmid vectors can be lowered by the elimination of transfer functions.

Other approaches can also be used to reduce the frequency at which the inserted DNA would be transferred from the recipient to other organisms, e.g. stable integration into the chromosome.

*Should not transfer any resistance markers to micro-organisms not known to acquire them naturally*

Frequently, genes for resistance to a variety of substances (e.g. antibiotics, heavy metals) are introduced for selection purposes into the recombinant organism. When evaluating a specific resistance gene the following should be considered:

- Whether and with what frequency the resistance marker(s) can be transferred from the recombinant organism to other organisms (see above).

- Whether such acquisition can compromise the use of a therapeutic agent or lead to environmental perturbations. Markers for substances such as antibiotics, not currently in commercial use should also be evaluated to determine whether the marker exhibits cross-reactivity or linked resistance.

- Whether selection pressure might exist for the specific marker. For example, selection in the environment of an organism carrying a resistance gene may be enhanced if the selecting agent in question is present in adequate concentration in the environment. This may occur, for example, as a result of the use of antibiotics in livestock feed, or of pollution by environmental contaminants such as heavy metals.

**r-DNA organism**

*Non--pathogenic*

The nature and, where appropriate, the source of the inserted genes must be considered. The type of gene product and its function must be examined in the context of the characteristics of the host. If, for instance, the gene product has no known role in pathogenicity and the host is not pathogenic, then the r-DNA organism is expected to be non-pathogenic.

*As safe in industrial setting as host organism or with limited survival, and without adverse consequences in the environment*

This includes safety to both man and the environment. In general, the approach taken should be to consider the nature of the host and to focus on the nature of the inserted genes and the resulting products. Their effects on biological fitness and adaptability, including attributes such as the ability to colonise new niches, should be taken into account. Adverse consequences can be avoided, for example, by using r-DNA organisms of limited survival in the environment in relation to the wild strain. In some cases it may be necessary to generate and/or collect data on specific properties, for example, through monitoring of environmental discharges.

## III. FUNDAMENTAL PRINCIPLES OF GOOD OCCUPATIONAL AND ENVIRONMENTAL SAFETY FOR PROCESSES USING GILSP ORGANISMS

The central objective is to identify appropriate good and prudent practices for handling GILSP and other low-risk organisms as described in Section I (pp. 8-9). These practices must be based on good principles of occupational hygiene and environmental management and on the use of physical controls where necessary.

Recombinant DNA-containing as well as other organisms used in industry will generally have been developed in the laboratory under the conditions specified by codes of good practice, guidelines or legislation governing research. Experience gained in using these organisms in the laboratory is one factor to be taken into account when determining the appropriate practices for large-scale production.

The fundamental principles of good occupational and environmental safety listed below should be applied for Good Industrial Large-Scale Practice, as well as for all levels of containment. These principles represent an attempt to describe the end to be achieved rather than an attempt to specify the technical means of implementation:

i) keep workplace and environmental exposure to any physical, chemical or biological agent including cellular products and debris to a level appropriate to the characteristics of the organism, the product and the process;

ii) exercise engineering control measures at source and to supplement these with appropriate personal protective clothing and equipment if necessary;

iii) test adequately, and maintain, control measures and equipment. The frequency of examination and testing will depend on the nature of the modified organism, the product and the process;

iv) test, as appropriate, for the presence of viable process organisms outside the process equipment, both in the workplace and in the environment;

v) ensure personnel have adequate training and experience;

vi) as required, to establish biological safety committees and/or consult with worker representatives and to consult with regulatory authorities;

vii) establish and implement a code of practice in the workplace for the safety of personnel and for the protection of the environment[3].

## NOTES

1. In Part One of this document the term "host" is used to describe the recipient organis m.

2. The concept of toxicity should not be limited to lethality, but should include mutagenicity, carcinogenicity neurotoxicity, etc.

3. The type of topics covered could include but are not limited to: prohibition of eating, drinking, smoking, mouth pipetting and application of cosmetics in the workplace; training and supervision of staff in safety and hygiene procedures; disposal of biological and other wastes; guidance for ancillary and maintenance staff; operation of bioprocessing and associated equipment; medical or health surveillance; incident response procedures.

*Annex*

## Suggested Criteria for r-DNA GILSP (Good Industrial Large-Scale Practice) Micro-organisms and Cell Cultures

(Revised Appendix F to *r-DNA Safety Considerations, 1986)*

| Host Organism | Vector/Insert | r-DNA Organism |
|---|---|---|
| Non-pathogenic | Well characterised and free from known harmful sequences | Non-pathogenic |
| No adventitious agents | Limited in size as much as possible to the DNA required to perform the intended function; should not increase the stability of the construct in the environment (unless that is a requirement of the intended function) | As safe in industrial setting as host organism, or with limited survival, and without adverse consequences in the environment |
| Extended history of safe use **OR** | Should be poorly mobilisable | |
| Built-in environmental limitations permitting optimal growth in industrial setting but limited survival without adverse consequences in the environment | Should not transfer any resistance markers to micro-organisms not known to acquire them naturally | |

*Part Two*

# GOOD DEVELOPMENTAL PRINCIPLES (GDP): GUIDANCE FOR THE DESIGN OF SMALL-SCALE RESEARCH WITH GENETICALLY MODIFIED PLANTS AND MICRO-ORGANISMS

## BACKGROUND

The 1986 OECD report *Recombinant-DNA Safety Considerations* concluded that "assessment of potential risks of organisms for environmental or agricultural applications is less developed than the assessment of potential risks for industrial applications". It went on to say that "the means for assessing r-DNA organisms can be approached by analogy with the existing data base gained from extensive use of traditionally modified organisms in agriculture and the environment generally". The 1986 report also suggested that because of "step-by-step assessment during the research and development process, the potential risk to the environment of the applications of r-DNA organisms should be minimised.

The recommendations in this area noted that "considerable data on the environmental and human health effects of living organisms exist and should be used to guide risk assessments", and that "research to improve the prediction, evaluation, and monitoring of the outcome of applications of r-DNA organisms should be encouraged". Any development of general international guidelines governing such applications was judged to be "premature" in 1986. It was recommended that "review of potential risks should be conducted on a case-by-case basis, prior to application. Case-by-case means an individual review of a proposal against assessment criteria which are relevant to the particular proposal; this is not intended to imply that every case will require review by a national or other authority since various classes of proposals may be excluded."

In April 1988, the OECD's Group of National Experts on Safety in Biotechnology met to consider the need for a follow-up programme to the 1986 report. The group decided that part of its programme would be to develop general principles that would identify a generic approach to the safety assessment of low -- or negligible risk small-scale field research. The principles, labelled Good Developmental Principles (GDP), would be developed while countries continued to use the general case-by-case approach as defined in the 1986 report.

At that meeting, there was agreement that GDP should apply equally well to both agricultural and other types of environmental testing (i.e. mineral leaching or waste degradation) and that a single document could appropriately describe principles for both these kinds of applications.

Given the importance and complexity of the subject, and its widespread interest, an earlier version of this part was made available for discussion and public comment in March 1990.

The present version results from the review and assessment of comments received, including those from environmentalists, industry, trade unions, the public and policymakers in general.

## 1. PURPOSE AND SCOPE OF THE REPORT

Part Two describes scientific principles for the design of small-scale field research with genetically modified plants and micro-organisms. The principles described, Good Developmental Principles (GDP), are intended as scientific guides to the performance of low -- or negligible risk small-scale field research, including basic and applied research. They are not intended to bypass or prejudice any regulatory action on field research with plants and micro-organisms. These principles will allow flexible national approaches to the design and conduct of small-scale field research.

Also addressed are plants and free-living and plant-associated micro-organisms. Future work may extend the application of GDP to other organisms as well as to animal vaccines.

## II. INTRODUCTION

In general, the progression in the development of a genetically modified organism[1] for use in the environment involves research conducted through a continuum of testing from laboratory to greenhouse/glasshouse to introduction into the environment. These stages can be represented as in the following diagram. In the research and development process, controlled experiments are conducted in properly designed facilities prior to release. Each stage may be revisited several times, e.g. to construct organisms with better field performance, or to accumulate additional data. In some situations, satisfactory results may be obtained in a single stage.

Codes of good practice or guidelines have been established, both nationally and internationally, for safe conduct of research in the first stage of the diagram, research in the laboratory/greenhouse/glasshouse. These primarily address human health and worker safety.

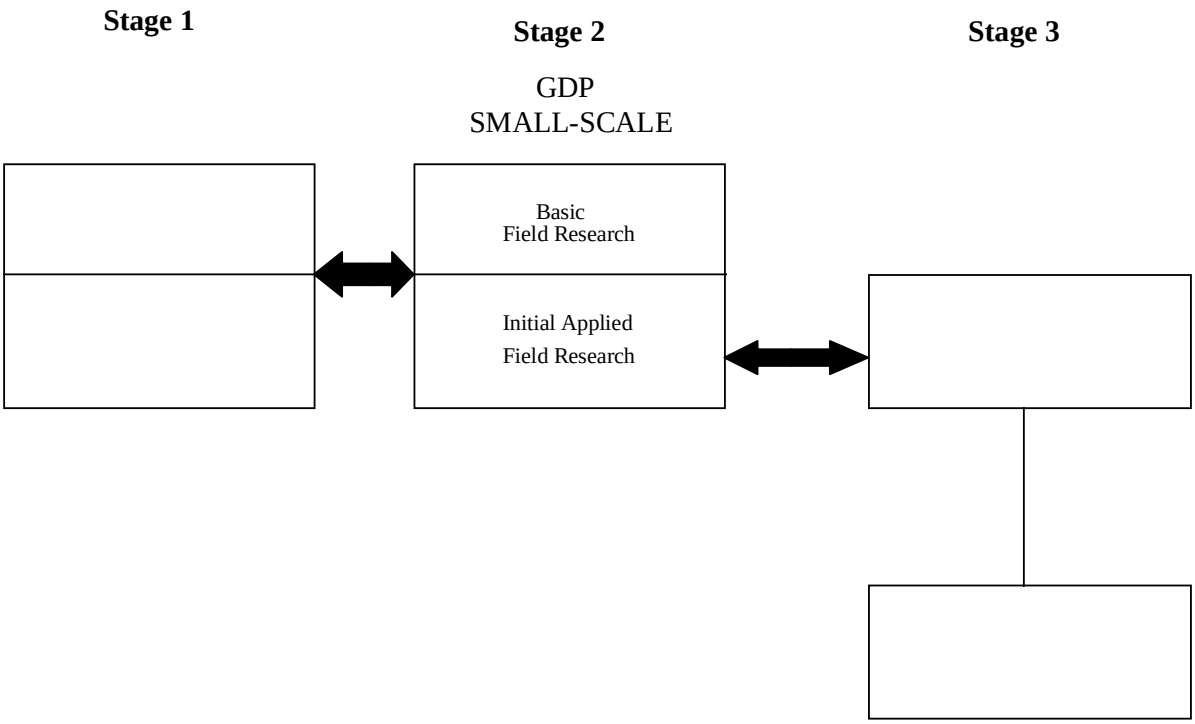
However, similar codes of practice and principles referring to environmental safety have not been compiled for the small-scale basic and applied field research stage (Stage 2).

This part presents general principles for the design of small-scale field research in Stage 2. The 1986 OECD publication *Recombinant-DNA Safety Considerations* provided, in Appendices, lists of considerations to be used in assessing field research involving genetically modified organisms. What follows describes how these considerations may be used in the design of low -- or negligible risk small-scale field research.

The application of GDP should help ensure the safety of small-scale field research with genetically modified organisms by providing guidance to investigators on selecting organisms, choosing the research site, and designing appropriate experimental conditions. It should assist in the review of proposals for small-scale field trials which in turn should provide data to predict the safety of large-scale trials as part of the step-by-step process. Annexes 1 and 2 (pp. 27 and 32) discuss the

interaction of experimental conditions with characteristics of plants and micro-organisms respectively.

Figure 1. **GDP in the context of field research**




## III. GOOD DEVELOPMENTAL PRINCIPLES (GPD): WORKING ASSUMPTIONS

The underlying assumption of GDP is that a set of general experimental principles can be identified under which small-scale[2] field research of low or negligible risk can be conducted with a specific genetically modified organism.

- The *first* working assumption is that certain general scientific principles related to the organism, the research site, and experimental conditions have varied relative importance in determining whether an experiment is of low or negligible risk.

- *The second* assumption is that a conclusion regarding that risk of an experiment can be reached by evaluating the relevant factors and their interaction under the conditions of the experiment including, when available, existing data from greenhouse and laboratory studies.

- The *third* assumption is that the interaction of these factors is easier to address in small-scale field experiments than in large-scale experiments because of their limited scope, which permits closer monitoring, generally easier assessment and analysis and

the possibility of more effective containment measures in the event of unforeseen and potentially damaging occurrences.

A number of assumptions are also made concerning the key factors which determine the safety of any specific experiment.

## IV. KEY SAFETY FACTORS

The key factors in determining the safety of any specific experiment are:

- the characteristics of the organism(s) used, including the introduced gene/genetic material;
- the characteristics of the research site and surrounding environment; and
- the use of appropriate experimental conditions.

### Characteristics of organisms

Certain organisms may have characteristics such that their use under a broad range of conditions would be considered to be of low or negligible risk. Other organisms with known adverse effects may be acceptable for field experiments provided the experimental design presents a situation in which it is possible to reduce the likelihood of these adverse effects by mitigation methods and/or confinement of the research organism or its genetic material to a restricted research site. However, it must be recognised that mitigation and confinement are more readily accomplished with higher plants than with most micro-organisms.

### Characteristics of the research site

The research site should be chosen both to design field trials of low or negligible risk, and to meet the objectives of the research. The term “site” is intended to include the research plot proper and an appropriate part of the surrounding environment.

The safety of research can be augmented by choosing a site comparable to one in which there is an extended history of relevant research and where dissemination and establishment have not been observed beyond the site.

At the small-scale stage of research, since the affected environment is generally more localised than at other stages, the investigator should be able to choose a research site most suitable from the safety aspect by identifying for example:

- important ecological and/or environmental considerations relative to safety in the specific geographical location (e.g. highwater table, heavy field run-off, etc.);
- climatic conditions;
- size, e.g. physical area;

- an appropriate geographical location in relation to proximity to specific biota that could be affected.

**Experimental conditions**

Scientifically acceptable and environmentally sound field research requires careful experimental design, e.g. formulation of an hypothesis and statement of objectives; development of specific methodologies for introduction of organisms, monitoring and mitigation; a precise description of the design of experiments, including planting density and treatment patterns; and description of specific data to be collected, and of methods for analysis to test for statistical significance.

The design of low -- or negligible risk small-scale fieid research includes: choosing an appropriate geographical location in relation to proximity to significant biota that could be affected; characterising the research site, including, for example, size and preparation, climatic features; designing introduction protocols including quantity and frequency of application; choosing methods of site preparation and cultivation; choosing methods for confinement, decontamination, monitoring and mitigation; designing treatments applicable to the research; developing suitable safety and handling procedures for application and contingency plans in the event of the need for early termination of an experiment.

Additional precautions may be required when considering a particular organism or trait or for particular environments such as aquatic environments.

Researchers designing and conducting these field experiments should carefully consider the following in the development of protocols and codes of practice for conducting small-scale field research with plants and micro-organisms:

1. Keep numbers of the modified organism to the lowest practicable level appropriate for the experiment.

2. Exercise measures to limit dispersal and establishment beyond the test site and supplement these measures when appropriate.

3. Monitor adequately the organism within the research site, both during the experiment and at its termination, and be prepared to apply control or mitigation measures if appropriate and necessary to avoid unintended adverse environmental effects during, at the termination of, or following the experiment.

4. Test for the presence of established organisms or, where appropriate, transferred genetic information, outside of the primary research site.

5. Apply control or mitigation measures if appropriate and necessary to avoid adverse environmental effects outside of the primary research site.

6. Develop procedures for termination of the experiment and waste disposal.

7. Provide appropriate safeguards/education and training for all personnel involved in research.

8. Maintain records regarding the results and conduct of their trials.

## V. APPLICATION OF GDP

### Experiments with plants[3]

The safety of small-scale field research with plants can be determined by analysing the characteristics of the organism and the research site and by designing appropriate, scientifically and environmentally acceptable, experimental conditions. The following discussion of GDP for plants includes characteristics of the organism and assumes the prudent choice of research site and experimental conditions.

The plants most likely to be tested are domesticated crop species. In many cases, there is extensive experience with their reproductive isolation and with the prevention of spread of plants outside the test area. Most domesticated crop plants cannot persist or thrive in non-cultivated environments.

Characteristics of plants to be considered include:

- the biology of the reproductive potential of the plant, such as its flowers, pollination requirements and seed characteristics, and an extended history of controllable reproduction with lack of dissemination and establishment in an environment comparable to the research site;
- the mode of action, persistence, and degradation of any newly acquired toxic compound;
- the nature of biological vectors used in transferring DNA to plants;
- interactions with other species and/or biological systems.

GDP should facilitate the design and conduct of field experiments so that: *i)* the experimental genetically modified plants remain reproductively isolated from the gene pool represented by sexually compatible plants outside the experimental site; **AND** *ii)* genes or genetically modified organisms will not be released into the environment beyond the research site; **OR** *iii)* plants are used which, even without reproductive isolation, will not cause unintended, uncontrolled adverse effects.

GDP can be applied in one or both of the following ways:

1. The experiment allows for the control of reproduction:
   - an experimental restriction or intrinsic biological limitation makes the plant incapable of reproduction;

**OR**

2. The experiment limits the likelihood of harm to (or significant impact on) the environment:

– there is minimal likelihood that the plant will survive, disperse, or become established beyond the research site;

**AND**

– any toxic compound newly acquired or enhanced by the plant has a minimallikelihood of detrimental effect on managed or natural ecosystems;

**AND/OR**

– gene transfer vectors that present a risk of injury, disease or damage to the plant have been adequately disarmed and/or eliminated from the plant.

The interaction of experimental conditions with the characteristics of plants is discussed in more detail in Annex I (p. 27). The scientific considerations described therein are derived from experience gained in field research with new plant varieties obtained by conventional and new plant breeding techniques.

**Experiments with micro-organisms**[4]

The safety of small-scale field research with micro-organisms can be determined by analysing the characteristics of the organism and the research site, and by designin 9 4p ,propriate scientific and environmentally acceptable experimental conditions.

As distinct from plants, tests with micro-organisms usually involve large populations, some portion of which may persist. The individual organisms in that population cannot always be genetically isolated, e.g. the possibility of horizontal DNA transfer p4nnot always be excluded in micro-organisms. Micro-organisms must be thought of in Statistical terms that consider the probability of an event occurring in a given population/ pnvironment.

Characteristics of micro-organisms to be considered include:
containment measures

– dispersal, survival and multiplication;

– interaction with other species and/or biological systems;

– potential for gene transfer;

– the mode of action, persistence and degradation of any newly acquired toxic compound.

GDP should facilitate the design and conduct of field experiments so that: *i*) transfer of genetic material of interest is controlled **AND** *ii*) dissemination[5] of micro-organisms containing that genetic material is controlled; *OR iii*) there are no unintended, uncontrolled adverse effects on other organisms even though transfer and dissemination may occur.

GDP can be applied in one or both of the following ways:

1. The experiment allows for control of transfer of genetic material and dissemination beyond the research site:

- The biology of the organism minimises the probability of horizontal gene transfer, or measures are taken to prevent or minimise it;

- The biology of the organism minimises the probability of horizontal gene transfer, or measures are taken to prevent or minimise it;

**AND**

- The organism has limited ability to compete;

**AND**

- Measures are taken to rninimise movement/dispersal of the micro-organism frorn the test site;

**OR**

- Measures are taken to prevent or mitigate establishment beyond the test site if necessary.

2. The experiment limits the likelihood of harm to (or sipfficant impact on) areas beyond the research site:

   - There should be no adverse environmental effects beyond the research site, even if the micro-organism should disseminate from the site, as shown by knowledge and previous experience (e.g. characteristics of the organism including the introduced gene/genetic material, environmental conditions, results from contained studies and previous field trials as assessed within the framework set out in *Recombinant-DNA Safety Considerations,* 1986 OECD report);

**AND**

The experiment should be designed to detect, as appropriate, for effects on other organisms (e.g. plant or animal health, microbial communities, ecosystem processes, other biological systems) and to control or mitigate such effects, as appropriate, should they occur.

The ability of a micro-organism to disseminate into the environment and to transfer genetic material to other organisms and the availability of suitable, reachable habitats/niches in the vicinity of the research site will, thus, be important factors in evaluating safety. The interaction of experimental conditions with the characteristics of micro-organisms are discussed in more detail in Annex 2 (p.32).

## NOTES

1. The term “Genetically modified organism” is employed here in a broad sense. Its scope may evolve over time with the progress of science and technology, and vary from country to country and agency to agency, depending on the various responsibilities and purposes involved.

2. In the context of this part, “small” refers to the minimum size required to fulfill the objectives of the experiment while maintaining GDP.

3. The principles developed here apply to gymnosperms and angiosperms. Principles for other plants including saprophyfic fungi have yet to be developed.

4. The principles developed here apply to micro-organisms which include: viruses, bacteria, microalgae, protozoa and fungi.

5. Dissemination comprises the concepts of “movement/dispersal” and “establishment” beyond the test site.

*Annex 1*

**Scientific Considerations for Small-Scale Field Research with Plants**

The following text describes scientific considerations underlying Good Developmental Principles (GDP) for field research with genetically modified plants. The size of field experimental plots will more than likely be determined by the characteristics of the experimental plants (i.e. orchard crops will require larger experimental plots, while grain crops could be adequately evaluated using smaller experimental plots). While selective plant breeding has been practised in some form for thoUnited Statesnds of years, it was after the rediscovery of Gregor Mendel's work in 1900 that the systematic breeding now practised by plant breeders became widely used. Observations made by scientists, based on a knowledge of plant genetics, plant morphology, plant reproductive biology and plant physiology, have resulted in the practices now used by plant breeders to ensure the genetic integrity of their experimental material. This experience and that gained from the controlled field tests of genetically modified plants help to identify plant characteristics and experimental conditions that allow the safe conduct of small-scale field research.

Small-scale field research with genetically modified plants is conceptually analogous to the small-scale field research already conducted by plant breeders in evaluating potentially useful new varieties. The genetic modifications achieved through conventional plant breeding techniques have produced single or multiple gene mutations and changes in chromosome number through: chemical treatment or ionising radiation; crosses between cultivars of a crop species; and interspecific crosses, including crosses between cultivated species and crosses between cultivated species and related non-cultivated species. When conducting conventional plant breeding research, attention is often given to preventing possible genetic influx from any sexually compatible plants into the research plot. It has not been demonstrated to date that natural transfer of genetic material from plants to organisms other than plants occurs.

Conventional small-scale field research evaluates the characteristics of a new plant variety and its interaction with the environment. Field experiments of new plant varieties produced by conventional plant breeding methods have shown that most new plants in breeding experiments are of no practical use to the breeder and are eliminated, with no further effect on either the environment or on subsequent plant breeding. Only a very small proportion of new germplasm lines produced by plant breeders warrants further research or eventual commercial release. This practice, however, does not imply that new plants are competitively unfit to survive in a variety of ecological niches.

There have been some instances where the intentional or accidental introduction of a foreign plant species into a new environment has had an adverse environmental impact. Examples include Johnsongrass (*Sorghum halepense*) introduced into South Carolina, United States, as a forage plant in the 1830s, water-hyacinth (*Eichhornia crassipes*) introduced into Florida, United States, as an aquatic ornament and the Asian weed kudzu (*Pueraria lobata*) introduced as a stabiliser of soil embankments and as crop forage on unproductive land. Many other important weeds (Canada thistle, yellow starthistle, field bindweed), now present in the United States, are the result of the accidental

introduction of foreign plant species. In Europe, there have been similar problems as a result of intentional or accidental introduction of foreign plant species such as sunflower (*Helianthus annuus*), common ragweed (*Ambrosia artemisiifolia*) and giant hogweed (*Heracleum mantagazzianum*) which causes severe dermatitis in man. These examples involve the uncontrolled release of a complete genome rather than the controlled transfer into plants of single or few genes which is the current case with genetically modified organisms. Therefore, the field testing of genetically modified plants conducted using GDP should not be considered analogous to uncontrolled introduction of foreign plants into entirely new environments, but experience from such introductions may provide relevant information.

**Reproductive isolation of genetically modified plants**

Conventional plant breeding experiments utilise reproductively isolated plants in the research plots in addition to limiting the size of the plots. Employing practices that ensure reproductive isolation of the modified plants is an excellent method for preventing dissemination of genetic material from the test plant into other members of the same or related species.

In considering natural mechanisms for reproductive or genetic isolation in the evolution of plant species, Stebbins (1950) emphasized those characteristics identified as “prezygotic” (occurring prior to mating), since they can usually be controlled by manipulating the experimental plants or the environment into which the plants are to be introduced. Plants manipulated in this way can be made incapable of producing and/or disseminating any genetic material (via pollen, seeds, etc.) that would allow new genes to become permanently incorporated in the gene pool of the species.

The practice of maintaining a considerable degree of reproductive isolation is currently used by plant breeders and by seed producers to produce genetically pure seed. In these practices, the emphasis is on preventing the contamination of the test or breeding plants with extraneous genetic material (in most cases via pollen) to maintain the genetic purity of the experimental or breeding plant population. Although the practices used to protect the genetic purity of a breeding line differ from those used in field research where the emphasis is on controlling dispersal of the genetic material of experimental plants from the test plot, the same principle of reproductive isolation applies. This principle can be applied successfully to reduce the likelihood of dispersal of genetic material from the experimental plot.

The practices currently employed by plant breeders and seed producers offer useful models for reproductive isolation in field research involving genetically modified plants. These practices result in the spatial, mechanical, temporal, and genetic isolation that evolutionary biologists use to define reproductively isolated plant populations. In most cases, if field research is conducted so that experimental genetically modified plants remained reproductively isolated from the pool of sexually compatible plants outside the experimental site, the objectives of GDP would be achieved. Using GDP, small-scale field research with genetically modified plants may be conducted with a reasonable assurance that it will have no significant adverse effect on the environment.

To provide some guidance in determining the types of practices that are appropriate for reproductive isolation, a list of examples is provided in the next paragraph. When reviewing these examples of practices currently used to achieve genetic isolation, consideration should be given as to how, in each instance, a particular practice compensates in some way for a characteristic of either the plant or the field research environment. The end result of using such practices should be that experimental genetically modified plants are reproductively isolated.

The following are examples of current experimental practices used to maintain reproductive isolation in plants:

- The most common method used to isolate plants from sexually compatible plant populations is spatial separation. Most requirements for growing pedigreed or certified seed include some specification as to the distance the field must be from any field containing plants of the same species. The specific distance required will depend on the biology of the species in question. Self-pollinated species with fragile pollen will require relatively short distances, while some open-pollinated species with hardy pollen will experience some degree of contamination when separated from compatible plants by as much as several miles.

- In the case of some plants, removal of the male or female reproductive structure(s) may allow plants to be safely grown in close proximity to compatible plants. An example of the use of this method is mechanical detasselling in seed corn production. By removing the tassel (containing the pollen-producing male flowers) it is possible to entirely eliminate the source of genetic material from the male that can be transferred via pollen.

- A variation of the technique discussed above involves the incorporation into the plants in question of a cytoplasmic male sterility trait. When this trait is present, almost no viable pollen is produced, and the plant will virtually remain reproductively and biologically isolated.

- It may be possible to grow the plants in question in such a way that flowering will occur either earlier or later than it would be expected to occur in plants of nearby compatible crops and/or wild plant species. This use of temporal reproductive isolation can potentially be as effective as spatial separation in limiting the movement of genetic material.

- Pollen dissemination may also be prevented by physical means such as covering of flowers (bagging) prior to anthesis.

- When the objectives of a field test do not require that seed be produced, as when forage qualities of alfalfa are being evaluated, it may be possible to harvest plants prior to flowering. In this case, reproductive isolation could be achieved in some crops that for some reason might otherwise be difficult to isolate.

Although reproductive isolation is likely to be the main safety concern for most small-scale field tests, there may be cases in which additional measures to ensure reproductive isolation as well as other factors should be considered. For example, the plants to be field tested may have been modified to contain or express toxins, or to contain biological vectors capable of transferring genetic material. The following two sections outline the nature of the problems that may be encountered in the cases of toxins and of some biological vectors, and provide factors to be evaluated when these types of field tests are anticipated.

**Plants genetically modified to contain or express toxins**

Many plants contain toxic compounds. Some serve as defenses against pathogens and predators. Genetic modification techniques can enhance or decrease a plant's defense mechanisms or can add new defense components to the plant. It may be desirable to develop plant varieties that contain toxic compounds or to cause toxic compounds native to the plant to be expressed at much higher than

naturally occurring levels. In many cases, field research involving plants expressing these toxins will be safe because enough will be known about an introduced toxin, its mode of action, the potential effects of the toxin on target and non-target organisms, and the techniques for incorporating the gene or genes coding for the toxin into the plant.

There is some possibility of environmental risk in small-scale field research involving plants modified to contain toxins, even if the plant genetic material remains confined to the experimental site. This is due to the fact that these plants might affect organisms entering the site (e.g. by making the toxin available to organisms not usually encountering the toxin in their ecosphere/niche) or have some residual, unintended effect on non-target organisms that are exposed to these plants or their products after the plants themselves have been removed from the field experiment site. It is possible to conduct research safely with plants genetically modified to contain some toxic compound or to express some native toxic compound at higher levels. There should be sufficient information about issues such as the mode of action, persistence, and degradation of the toxin to be able to limit the effects of the toxin to the target organisms at the test site. Additional precautions may be as simple as fencing the site, or as complex as planting the test plot at an isolated location, caging the plants involved in the field test, or instituting strict measures to account for all plant material produced in the field research.

**Plants genetically modified through the use of biological vector systems**

Various physical, chemical, and biological means are available to transform plants with new genetic material. These techniques include the use of electroporation, micro-injection, ballistic microprojectiles, organisms or molecular vectors. The first three techniques are mechanical procedures that are unlikely to increase the probability of inadvertent transfer of genetic material at any time other than at the initial insertion. However, there is the possibility that the vector could subsequently act as an infectious agent unless the vector becomes biologically inactive and/or is eliminated from the transformed plant.

The safety of small-scale field research with plants that have been transformed through the use of biological vectors is enhanced when the vector system is unlikely to transfer genetic material after the initial transformation has occurred. If the vector presents a plant pest risk (i.e. a risk of injury, disease, or damage), that risk must be adequately eliminated. In most cases the vector should be eliminated from the plant or inactivated once the transformation has been completed. DNA that is to be used in developing a genetically modified plant should be: *i*) well characterised and unlikely to be transmitted after entering the plant (disarmed *Agrobacterium tumefaciens* Ti plasmid meets this specification); and *ii*) transferred from the same or closely related species (as the recipient plant); and/or *iii*) transferred from non-pathogenic prokaryotes or non-pathogenic lower eukaryotic plants; and/or *iv*) transferred from plant pathogens only if the sequences capable of producing disease or damage in plants have been deleted.

Currently, the vector system most widely used to transfer DNA into a plant cell is naturally present in the bacterium *Agrobacterium tumefaciens* and is commonly referred to as the Ti plasmid. There is now a considerable body of evidence based on experiments conducted under laboratory and greenhouse/glasshouse conditions establishing the safety of this vector system. In most of the field research with genetically modified plants conducted to date, the vector systems derived from *A. tumefaciens* have had the genes associated with the pathological response to infection physically deleted. In addition, the transformations have been conducted in such a way that no vector sequences involved in pathogenicity, except the border sequences, are present in the transformed plant, and the

vector agent, the bacterium, does not survive. In this way, the possibility of the vector being able to cause any transfer of genetic material from the modified plant has been eliminated (see Section V, p. 23).

*Annex 2*

**Scientific Considerations for Small-Scale Field Research with Micro-organisms**

The following sections describe the scientific considerations underlying Good Developmental Principles (GDP) for field research with genetically modified micro-organisms. Small-scale field research presents a situation where the issues to be addressed are constrained by the relatively small size of the experimental plot. Such research would normally occur at only a single or a few geographic locations as opposed to large-scale testing or use, or to unlimited application. The results of research on biological control agents as a means of controlling agricultural pests indicate that the scale and frequency of introduction appear to be important factors in determining whether the micro-organism will become established and what the effect of the introduced micro-organism on the environment will be.

In a limited small-scale field experiment, the potentially affected environment is, in general, localised, and it is therefore easier to identify the important ecological/environmental considerations that should be evaluated to devise a safe experiment. Moreover, because of the small size of the experiment, procedures and experimental design to confine the experimental organisms may be effectively used.

**Application in the environment**

The methods for applying the organism and the amount of inoculum are important considerations in determining the safety of field research. "The location and nature of the site of application, and the magnitude of the application are important for assessing safety" (OECD, 1986).

Micro-organisms are generally applied in small-scale field research as soil amendments, as foliar sprays, as seed treatments, or as inocula introduced into the vascular tissues of plants. While organisms may be introduced using other methods, the process for evaluating relevant safety considerations is expected to be similar in most cases. Therefore, the discussion of scientific principles can focus on these few as the most commonly used.

Greater dispersion of the micro-organisms from the field plot would be expected with those application methods that involve creation of aerosols. Consequently, relatively larger border areas (buffer strips of land) might be part of the field research design for an experiment involving foliar sprays. Alternatively, aerosol formation may be minimised by the choice of drip application and drip irrigation, rather than spray applications and spray irrigation.

**Dissemination, including survival and multiplication, in the environment**

"The relative ability of the organism to survive and multiply in the environment in which it is applied and to be disseminated to new environments is an important consideration for assessing the safety of the release" (OECD, 1986).

Most of the data that form the basis for a discussion of the following considerations and, consequently, for the development of an appropriate field research design, are based on principles derived from the studies of a few micro-organisms. Limited information is available on the dissemination of saprophytic organisms (except for some that interact with plant pathogens, e.g. *Agrobacterium radiobacter*).

These studies show that dissemination depends on three factors: *i*) the inoculum (size, fitness, infectivity, viability); *ii*) the movement[1]/dispersal[2] properties of the population; and *iii*) the availability of suitable habitats or niches. "Dissemination" is composed of the concepts of "movement/dispersal" and "establishment". "Establishment" encompasses "survival and multiplication", as well as "movement/dispersal."

In evaluating field research, it is not possible to separate completely the concept of "dispersal" from the concept of "establishment". Rather, these concepts must be considered in concert. For example, if it is accepted that an organism will not become established, dispersal from the experimental plot would be of lesser concern, and methods of controlling dispersal would assume a position of lesser importance. On the other hand, if dispersal from the experiment plot is low, either because of the characteristics of the experimental organism or because measures to control movement/dispersal have been implemented, the probability of establishment may be less.

***Inoculum***

The survival of an experimental micro-organism is dependent on a number of factors. At this time, it is not possible to describe all the factors influencing the rate of growth of a micro-organism in the environment. However, some prediction of likely behaviour can be made based on existing knowledge and empirical observations generated from a number of sources: greenhouse testing, microcosm testing, knowledge of the behaviour of closely related organisms (e.g. parental organisms and the intended function of the introduced trait if the experimental organism is genetically modified).

An inoculum must contain sufficiently high numbers of micro-organisms at the research site in order that a minimum level is present for effective dispersal to other sites. In addition, it is likely that some dilution of inocula will occur as the micro-organism leaves the research site. Dilution would probably increase as the micro-organism moves further from the test site without encountering a suitable habitat. These assumptions appear to be supported by plant pathology studies which have shown that dissemination is directly proportional to the size of the source-pool (in this discussion, the source-pool is considered to be equivalent to the number of micro-organisms of the test strain in the original research site).

It should be noted that the number constituting a minimum effective inoculum can vary considerably from organism to organism, and thus no single standard number of organisms can be cited as a minimum effective inoculum. Dispersal by vectors or by mechanical transport may lower the minimum effective inoculum load; this must be taken into account. It can be assumed that for some organisms, a small number of organisms would be an effective inoculum, while for other

organisms very large numbers are necessary. In some cases, for example, competition or other pressures (e.g. predation) can be overcome only by a large incoming population. What would constitute a minimum effective inoculum must, therefore, be determined on a case-by-case basis.

Instituting measures to lower the number of micro-organisms leaving the research site, however, would lower the probability that a number of organisms sufficient for a minimum effective inoculum would arrive at other sites. An experimental plan designed to use such measures can be implemented for small-scale field research.

***Movement, dispersal and transport***

The rate of dissemination is extremely sensitive to the effectiveness of movement/dispersal. It appears that, in general, the more effective movement/dispersal, the faster dissemination can occur.

Effectiveness of movement/dispersal generally depends on several factors. These include: mode of movement/dispersal mechanism of achieving transport (including ability to adhere to soil or other particles); ability to infect vectors; ability to adhere to potential means of mechanical transport (e.g. animals, humans and their tools); ability to survive transport. These factors are dependent on the biological characteristics of the experimental organism. Therefore, biological characteristics of the test micro-organism must be considered in evaluating the safety of field research.

While some micro-organisms are dispersed by several means, others may be restricted to one or a few modes of movement. In general, the more highly adapted a micro-organism is to movement by one route, the poorer are its chances of movement by other routes. An understanding of potential routes of movement/dispersal and knowledge and implementation of methods of limiting movement/dispersal along these routes can be used to design safe field research and underlines the need for monitoring.

Micro-organisms are transported by a variety of routes as described below: *i*) by wind; *ii*) by water; *iii*) by mechanical means (e.g. humans and animals); and *iv*) by biological vectors.

*i) Wind*

Effectiveness of aerial dispersal is influenced by several factors. These include: mechanisms of entering the atmosphere (take-off), particle shape, ability to survive environmental stress (e.g. desiccation, uv light), ability to adhere to soil and other particles. Some micro-organisms have adaptations which permit them to disperse aerially.

These adaptations are diverse, varying from passive processes such as being shed under gravity to being propelled long distances. Other micro-organisms are dispersed aerially through passive means, e.g. some micro-organisms adhere to soil particles. Rafts of soil or dust particles are raised by wind when the ground is heated by solar radiation. The micro-organisms attached to these soil particles are transported as the soil is blown by the wind. Some micro-organisms adhere to insects or mites which can then be dispersed by wind currents.

The positioning of a field research plot can be used to address and limit potential transport through the aerial route. For example, consideration can be given to situating the experimental site so that natural features of the landscape such as trees, hills, windbreaks, or fences can be used to

influence wind currents. When the test micro-organism possesses a high potential for dispersal by the aerial route, the positioning of the small-scale research plot on an off-shore island may provide acceptable security.

*ii) Water*

In water, dispersal is influenced primarily by the transport properties of the suspending medium. Thus, the hydrology of soil water and groundwater flow and the proximity of open bodies of water (e.g. lakes, rivers, streams) and water supplies for irrigation are among the primary physical determinants of water-borne dispersal from a terrestrial experimental plot.

Rain or irrigation water can also serve as a means of transport. Bacteria, viruses, and spores, sclerotia, and mycelial fragments of fungi can be dispersed by rain or irrigation water that washes the surfaces of plants or moves over or through the soil.

Rain splashes can throw droplets, potentially micro-organism-laden, from plant surfaces into the air. Splash dispersal occurs when water droplets impinge on plant surfaces covered with micro-organisms; for example, certain plant pathogenic bacteria can be spread for kilometres by driving rain.

The research plot can be designed to address and limit dispersal through these potential routes. For example, border strips around the research site can be used to isolate plants within the research plot and thus prevent micro-organisms contained in splash-generated droplets from encountering suitable habitats proximal to the test plot. Design features such as avoidance of an overhead irrigation system or the inclusion of tile drains with suitable decontamination systems in the test plot can be implemented.

Moreover, the research plot can be situated so as to limit access of the test micro-organism to groundwater or open bodies of water under both average and exceptional climatic conditions, and it is possible to control water flow through the use of drainage, collection and physical barriers.

*iii) Mechanical means*

*Human activities*: Humans disperse all kinds of micro-organisms over short and long distances in a variety of ways: through the successive handling of plants, through the use of contaminated tools and other equipment, through the transport of contaminated soil, plants, seeds and nursery stock.

Mechanical disturbances such as tillage may loft “rafts” of soil bearing clumps of micro-organisms into the air. These rafts may then settle downwind of the test plot. Likewise, any activity that generates aerosols can also create a potential route of dispersal for micro-organisms contained in the aerosol droplet.

In small-scale field research, care can be taken to limit dispersal of micro-organisms by human activities. For example, access to the test plot can be restricted to those individuals trained in procedures appropriate for limiting dispersal. Mechanical disturbances can be limited in a number of ways, such as by the choice of crop (e.g. no-till varieties) or procedures. Finally, the transport of micro-organisms on contaminated materials can be restricted by use of appropriate decontamination procedures.

*Animals*: In nature, a variety of animals may come into contact with and serve as vectors for micro-organisms. For example, bacteria may be transported by browsing and burrowing mammals, soil arthropods, earthworms, and soil clods adhering to duck feet.

In small-scale field research, appropriate measures can be taken to limit the access of animals to the test area. This might include, for example, screening or fencing of the experimental site. Maintenance of such physical barriers is essential, as is their continued monitoring, to ensure their effectiveness.

*Other*: Insects can transport micro-organisms phoretically. Their bodies can become smeared with bacteria or sticky fungal spores, and as they move among plants, the insects carry the micro-organisms on the surfaces of their bodies from plant to plant. The micro-organisms are then deposited on plant surfaces or in the wounds that insects make on the plants during feeding. Wounding often leads to higher establishment efficiency.

There are other methods by which passive dispersal can occur. For example, micro-organisms that colonise flowers and buds may be dispersed by plant pollen. Because fungi and bacteria are closely associated on plant surfaces, contamination of fungal propagules by bacteria is possible and may be a means of passive aerial dispersal for bacteria.

These types of potential vectors can frequently be addressed by the experimental design of the field test. For example, as noted in the section dealing with plants, a number of methods of dealing with pollen production and dispersal are available.

*iv) Biological vectors*

Micro-organisms can be transmitted by insects during feeding and movement of the insect from plant to plant. By definition, the insect vector and the micro-organism establish a specific relationship. A vector carries the micro-organism from one place to another and deposits it effectively (usually through wounding of the plant) where it can become established. Although there are a few exceptions, the more highly adapted and specific the vector/micro-organism relationship, the less likely in general the micro-organisms will be moved by other vectors.

The relationship between the vector and the micro-organism can be either persistent (circulative and propagative) or non-persistent. The persistent or circulative type of vector/micro-organism relationship occurs when the insect is able to transmit the micro-organism over an extended period of time and the micro-organism can multiply in the insect. Non-persistence refers to a relationship in which the vector acquires the micro-organism after a short feeding period on the plant, can transmit the agent to another plant immediately after feeding and then rapidly (within minutes) loses the micro-organism.

The common insect vectors are aphids and leafhoppers, but white flies, mealy bugs, beetles, dipterans, psyllids, thrips, mites and others have also been documented as vectors. Aphids and leafhoppers are by far the most important vectors of plant viruses and mycoplasmas (bacteria without cell walls).

Insects can vector micro-organisms for both short and long distances. Insects like leafhoppers are strong fliers. Some anthropods, such as mites, cannot fly but can be carried passively by wind.

Even insects which are not strong fliers can disperse micro-organisms over long distances since these airborne insects can be carried hundreds of kilometres by wind.

Small-scale field research design can be used to address potential vectoring of test micro-organism by insects. For example, if it is known that the test micro-organism is transmitted by aphids, a judicious choice of site might locate the test at an altitude where aphids are not present or when the aphid population is low. Using aphid repellents or denying vectors access to plants by netting are methods that could also be employed in the experimental design. These arrangements are rarely totally effective in eliminating vector activity and are highly dependent on the climatic situation of the particular season concerned.

***Availability of suitable habitats***

One of the most important considerations in determining whether a micro-organism will be disseminated is whether habitats[3] and/or niches[4] in which the micro-organism will become established are available.

The distribution and number of potential habitats in an area to which the micro-organism may be moved/dispersed are important determinants of establishment. The number, distribution, size, and susceptibility of the habitats influence the probability that a micro-organism will be successful in encountering and establishing itself in suitable habitats.

If the density of potential habitats is low and the habitats are separated by relatively large distances, the probability of successful dissemination is greatly reduced and indeed may approach zero. Strategies based on habitat density are used in agriculture to control pathogen dissemination. For example, fields can be planted with "multilines" of a crop. "Multilines" consist of several different varieties of the crop species with each variety possessing a different gene for resistance to the pathogen. Since the pathogen does not find a sufficient density of suitable habitats (susceptible plants), it does not disseminate in an epidemic fashion. When such strategies are employed, the micro-organism may proliferate within the experimental plot, but it would not disseminate outside the plot if it does not find suitable hosts.

Experimental design in a small-scale field trials can be used to address, to some extent, the issue of density and distribution of potential habitats. For example, test site locations may be selected based on the distribution and size of likely potential habitats in the experimental region. This is termed "geographic isolation".

Other strategies may be employed in the area proximal to the research site to help limit potential suitable habitats and thus control dissemination. For example, in one recent field experiment involving a Rhizobium species, wild leguminous plants which might have been suitable hosts/suitable habitats were removed from a 50 metre radius of land surrounding the research site. However, such procedures require thorough monitoring as most soils contain weed-seed banks capable of germinating.

***Multiplication and survival***

As noted in the previous section, survival and multiplication of the experimental micro-organism are important to producing a sufficiently large source-pool to permit dissemination. In order to

increase its numbers at the site of introduction, the experimental micro-organism must be able to compete effectively against other organisms in the research site, or find a new niche without competitors or containing less effective competitors.

Clearly, in attempting to evaluate the probability that an introduced micro-organism will be an effective competitor, be favoured by selection or find a new niche, a number of factors should be examined. These include: the source of the test organism and the source of the added gene, if any, and the environment in which the test will occur. In many instances, the micro-organism will be experimented with in the agro-ecosystern from which it or its parental micro-organisms were isolated. In such a situation, neither the introduced gene nor the introduced micro-organism will be new or unique in that environment, although the frequency at which the gene/micro-organism combination occurs in that site subsequent to application may differ from that generally observed.

The added gene/micro-organism combination would be in competition with the indigenous population of micro-organisms. While this does not guarantee that the added gene/micro-organism combination will not be an effective competitor in the test environment, it does set some limit on the types of risk scenarios to be considered. In this type of research situation, a knowledge of the function of the added gene and of the behaviour of the parental organisms can be used to predict the likely response of the gene/micro-organism combination to factors such as competition for nutrients, predation and environmental stress, selection, and antibiosis.

Given present knowledge, however, it is usually only through actual field trials that behaviour can be assessed, and the competitive ability of the experimental micro-organism will frequently have to be tested empirically. Data generated in the laboratory, greenhouse/glasshouse or microcosm may, in addition, form an important element in an evaluation of small-scale field research.

That the inoculum used in limited small-scale field research is frequently insignificant when compared to the indigenous population also plays a role in determining the likely fate of the gene/micro-organism combination. When relatively small numbers of the gene/micro-organism combination are added to an experimental site, it is probable that the competitive advantage lies with the indigenous population. In addition, when the application involves a relatively small number of organisms, the probability that sufficient genetic variation will exist in the inoculum from which genotypes can be selected is less.

In some instances, the micro-organism or the added gene may originally be isolated from environments other than the environment of the research site. In this situation, a careful comparison of competitive ability of the gene/micro-organism combination can be based on research in controlled environments such as greenhouses/glasshouses, microcosms, etc. The intended function of the added gene and the behaviour of the recipient parental micro-organism are also important considerations. An appropriate environmental design would take into account these considerations.

Competition and selection are important considerations in evaluating a submission and designing safe small-scale field research. The phenomenon of “finding a new niche” will be treated here as a facet of selection.

*i) Competition*

Negative interactions within a microbial community in a habitat are termed “competition”. Competition is used here in a broad sense to include competition for available substrates and other

negative interactions such as those resulting from production of toxic substances. Competition occurs when several populations are striving for the same resource, whether it be space, light, hosts, etc., or a limiting nutrient. In natural habitats with very low concentrations of available substrates, intense competition occurs.

*Free-living soil micro-organisms*: Most of the information on free-living soil micro-organisms is derived from experience with *Rhizobium* species and microbial amendments used as biological control agents. This experience shows that at the end of the growing season, the added micro-organism does not usually predominate. To explain these observations, it has been hypothesised that the organisms of the microbial amendment must compete with an indigenous flora well adapted to local conditions and are not always effective in this competition. This pattern of competition may differ with micro-organisms that have significant resting spores, such as many soil-borne fungal pathogens and semi-saprophytes.

A micro-organism must contend with numerous factors when it is placed in the soil environment. These include: a number of well-adapted competitors (since soil is a complex matrix in which various types of organisms abound); environmental stresses (e.g. chemicals, water and temperature); various levels of predation; competition for resources; and antibiosis.

Micro-organisms proliferate when nutrients are available and temperature and moisture levels are adequate. However, even when nutrients are in abundance, soil inhabitants must compete for them. In a situation of relative abundance, the competitive advantage lies with those having the highest growth rate. The more frequent situation is that nutrients are scarce, and organisms must frequently survive long periods of starvation. In this situation, populations with the greatest ability to survive stress conditions will generally have the competitive edge. Organisms that produce resistant structures (e.g. spores and sclerotia) are best adapted to survive the adverse conditions resulting from long periods of environmental stress and starvation. Some species have developed strategies through which they can survive for long periods of time as dormant vegetative cells.

Antibiosis occurs when one microbial population produces a substance that is inhibitory to other populations. Examples of antibiosis include production of substrates to suppress competitors and production of substances such as lactic or sulfuric acid, alcohol, acetic acid, and low-weight organic acids. Antimicrobial agents probably have a significant function in competitive interaction in micro-environments. The complementary competitive strategy would be possession of an inherent resistance to antibiotics produced by other organisms. Bacteriocins and biological toxins may also suppress populations of phytopathogens in the soil, and microbial strategies to deal with these substances probably exist.

Predation may also be a factor influencing microbial survival and population levels. Free-living nematodes, protozoans and bacteria act as predators on micro-organisms in the soil. Although the impact of such predators on microbial populations is unclear, it is likely that micro-organisms have developed strategies for dealing with predation.

Soil is a complex matrix presenting a highly competitive environment. The interplay of the factors described above and the response of the species to them create a balance of life in the soil which will affect the comparative competitive ability of the applied micro-organism.

*Host obligate micro-organisms*: Micro-organisms that depend on a host for survival are termed in this paper host obligate micro-organisms. Most available information addressing the factors

affecting competitive ability in host obligate micro-organisms was generated from studies of micro-organisms as biological control agents, and in plant pathology as well as in plant breeding.

In the micro-organism/plant interaction, host obligate micro-organisms may be epiphytic (on the surface of the plant) or endophytic (inside plant tissues) or both.

The endophytes have few competitors (other plant pathogens or possibly secondary invaders of diseased tissue), when compared to the epiphytes or free-living soil micro-organisms. Endophytes such as viruses, viroids and some prokaryotes (e.g. rickettsia-like bacteria, mycoplasmas and spiroplasmas) multiply entirely within their host or vector and generally are considered labile when exposed to the outside environment. There are, however, certain types of plant viruses that are known to survive in water, soil and crop debris. The environment in which they must compete is, thus, determined to a great extent by the host. Although they may have fewer microbial competitors, endophytes must deal with host defenses.

Plant obligate epiphytic micro-organisms may be categorised on the basis of the kind of nutritional relationship they maintain with the host. In their residency or epiphytic phase on leaves or roots, certain host obligate micro-organisms exist mainly if not entirely in an apparent state of commensalism with the plant. They obtain nutrients (as leaf or root exudates) from the plant but cause no harm to it. However, given the right conditions, they can kill and destroy host tissues through the action of toxins and enzymes and then multiply in the dead tissue.

In a second type of nutritional relationship, the host-obligate micro-organism obtains nutrients from a plant by killing the host tissue in advance of colonisation.

Many of the factors affecting competition among free-living soil micro-organisms can be seen in host-obligate micro-organisms. These include competition for space, competition for nutrients, predation, environmental stress, and antibiosis. In addition to dealing with these factors, both epiphytic and endophytic host-obligate micro-organisms must also find and colonise/infect suitable hosts. The need for host-obligate micro-organisms to find suitable hosts is a factor which can be used in designing an experimental protocol to test these organisms safely.

*ii) Selection*

Selective pressure is exerted by the environment and favours organisms possessing adaptive features. The best known example of selection in micro-organisms is the emergence of bacterial strains resistant to antibiotics. Selection of resistant strains is promoted by the use of antibiotics for treatment of human and animal microbial infections, in animal feed, and for agricultural purposes. Another example of selection is the increase in the numbers of micro-organisms capable of degrading certain man-made synthetic organic compounds (e.g. pesticides). In this instance, selection is promoted by the introduction of large amounts of these man-made compounds into the environment.

For the purposes of this part, “discovery of a new niche” is treated as a form of selection. It occurs when a micro-organism develops the capability to perform a “new” function within an ecosystem. It can also occur when a micro-organism performing a function which the indigenous community does not perform is introduced into an ecosystem.

Selective pressures affect the ability of an organism to survive, multiply and increase its relative proportion of the community. Selection thus can have an important influence on movement/dispersal and establishment, as well as on survival and multiplication.

**Interaction of the Micro-organism with other Species and/or Biological Systems in the Environment**

Experimental micro-organisms in small-scale field research can interact with other species in a number of ways. In Recombinant-DNA Safety Considerations, two specific kinds of interaction are noted in the outline. These are: *i*) the effects of the micro-organisms on target or non-target organisms; and *ii*) the potential for and effect of horizontal transfer of genetic material. The following paragraphs address these two types of interaction, and an initial attempt is made to examine them. These considerations can also be related to field research design.

***Target or non-target organisms***

Many of the micro-organisms that are tested in field plots are intended to have effects on another organism, the target organism. For decades, plant pathologists have used micro-organisms that cause plant disease in the field to evaluate plants for disease resistance. Other plant pathogens have been tested in the field to gain fundamental knowledge about the biology and the pathogenicity of those micro-organisms. Micro-organisms used as biocontrol agents are specifically selected or modified to affect a target pest organism. Some micro-organisms such as *Bacillus thuringiensis* are used routinely in the environment as biological control agents for some lepidopteran insects. Research using unmodified micro-organisms has been conducted with little adverse effect on the environment even though the micro-organisms have known effects on other organisms in the environment being reported on. The issues that are routinely considered in these tests are instructive in testing genetically modified micro-organisms.

When a micro-organism is experimented with, it is important not only to evaluate the expected effect on the target organism but also the effects on non-target organisms. When genetic engineering is used to modify micro-organisms to act as biological control agents, the genes that are inserted may encode toxins or they may broaden the host range or increase virulence on the micro-organism for a particular target organism. The effect of any new trait on the host range of the micro-organism should be evaluated before field testing. Potential non-target organisms should be identified by experimenting with representative species under contained conditions. It is generally unlikely that the relative abundance of a species in a community or ecosystem will be significantly altered as a consequence of small-scale field research if the micro-organism can be effectively limited to the plot and its immediate surroundings. Yet it is important that field research be conducted so as to limit exposure to sensitive non-target species.

These concepts call be applied to specific examples. New strains of *B. thuringiensis* should be experimented with on a plot on which no threatened or endangered species of lepidopteran insects will be exposed to the delta-endotoxin produced by the bacterium. It is essential that great care be taken in testing beneficial insects for sensitivity to the test micro-organism and in limiting the exposure of significant populations of sensitive beneficial insects.

***Gene transfer***

The gene transfer capability of an engineered micro-organism or the stability of the genetic construct may affect the micro-organism's interaction with other micro-organisms. Gene transfer refers to the dissemination of genetic material through natural genetic mechanisms.

The factors to be considered in analysing the effects of gene transfer on the safety of a genetically modified micro-organism are the following:

1. What is the probability of horizontal transfer of the genetic material?

2. If the gene is transferred, will the new genetic information be maintained and expressed?

3. What is the known function of the new genetic material?

4. If the modified micro-organism moves beyond the point of introduction, how will it affect, as a result of the transformation, the surrounding populations or communities of plants, animals, and indigenous microbes?

Gene transfer refers to the dissemination of genetic material through natural genetic mechanisms. The mechanisms by which plasmids and/or chromosomal genes are transferred include conjugation, transformation, transduction, and cell fusion. Although these mechanisms have been studied in the laboratory, little is known about the frequency of genetic exchange in nature. We expect that genetic transfer frequencies are lower in nature compared to the laboratory, but frequencies in nature have not been extensively studied. A few exchanges of genetic material in nature or simulated natural settings have been documented.

Several factors that may affect transfer are the presence or absence of: *i*) large bacterial densities that enhance mating; *ii*) free DNA that may promote transformation; and *iii*) clay materials or minerals that may promote growth and plasmid transfer but not transduction. The presence of wide host-range, high copy number plasmids may provide more opportunity for dispersal, and relatively large numbers of donor cells facilitate transfer to recipients. In addition, other factors that affect transfer are spatial, temporal, and physiological separation of bacteria; immobilisation through adhesion to soil particles, organic materials, and other living organisms; genetic barriers such as restriction systems and plasmid incompatibility; and environmental conditions.

On the basis of similar considerations, estimates have been made of the transfer frequencies likely to be observed in specific environments. However, the frequencies at which genetic transfer is likely to occur and the significance of such transfer, in comparison to transfers which occur in nature, will, for the moment, have to be evaluated on a case-by-case basis.

## NOTES

1. "Movement" refers to an active process that may involve behavioural choices such as a relationship with an insect vector.

2. "Dispersal" refers to a passive process such as rain splash.

3. A "habitat" is the physical location where an organism is found. The physical and chemical characteristics of habitats influence the growth, activities, interaction, and survival of the micro-organisms found in them.

4. A "niche" is broader than a habitat. A niche describes not only the physical habitat but also the functional role and the actions of the micro-organisms within that space. As used in this document, the term "niche" describes a functional role of an organism within an ecosystem.

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**Centraalbureau voor Schimmelcultures**
Fungal Biodiversity Centre
*Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW)*

# 1. Report: Identification of *Aspergillus luchuensis* FL100SC strain and analysis of secondary metabolites

**Utrecht, October 18 2013**

**Study Code: Det 13.094B**

Study Sponsor:

Advanced Enzyme Technologies Ltd

Plot No. A-61162, MIDC Malegaon, Tuluka

Sinnar Dist., Nashik-422 113, Maharashtra, India

Contact:

Dr. Anil Kumar Gupta

AET ref: Letter dd. August 2 2013

## 2. Table of contents

## 3. Summary

The objective of this study was to identify strain FL100SC provided by M/s Advanced Enzyme Technologies Ltd India and analyse for presence of toxic metabolites.

Analysis of the sequence data (partial fragment BT and partial fragment CMD) allowed the identification of strain FL100SC as *Aspergillus luchuensis*. No known mycotoxins were detected in the analysed extract.

**4. Quality statement**

The procedures described in this report are carried out in compliance with current quality standards for EU food additives and laboratory practices.

Utrecht, October 18 2013

(b) (6)

Dr. R.A. Samson

(b) (6)

Ing. M. Meijer

**5. Key study personnel**

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**6. Objective**

The objective of this study was to identify *Aspergillus luchuensis* FL100SC and to carry out toxic metabolite analysis, if any, produced by the strain.

**7. Test product**

The following strain was examined: FL100SC *Aspergillus luchuensis*. This strain was cultivated and extracted at the CBS Fungal Biodiversity Centre in Utrecht. The extracts were sent to Harm Janssens (T.L.R. international laboratories b.v.), who tested the presence of metabolites and compared them with all potential significant toxins.

**8. Methods**

**8.1. Metabolite analyses**

The strain was cultured on optimal media for the production of secondary metabolites: Malt Extract Agar (MEA), Yeast Extract Sycrose agar (YES) and Czapek Yeast extract Agar (CYA) (Frisvad and Filtenborg, 1989; Frisvad and Thrane, 1993; Frisvad et al, 2007). The isolate was incubated for 7 and 14 days in darkness at 25°C. For extrolite analysis three agar plugs with fungal colonies of each plate were combined and extracted using the organic solvents dichlormethane:methanol:ethylacetate (2:1:3) with 0.5% formic acid was used (Smedsgaard, 1997). The samples were subsequently analysed by high performance liquid chromatography (HPLC) with diode array detection (DAD) and Time-of-flight mass spectrometry (TOF-MS) (Frisvad and Thrane, 1993; Nielsen and Smedsgaard, 2003). The metabolites found were compared to a library made from authentic standards run at the same conditions.

### 8.2. Identification

DNA from the strain was extracted from the mycelium using the Mo-Bio laboratories; UltraClean Microbial DNA Isolation Kit. Fragments containing of part of the β-tubulin gene (BT) was performed using the primers Bt2a (GGTAACCAAATCGGTGCTGCTTTC) and Bt2b (ACCCTCAGTGTAGTGACCCTTGGC). Fragments containing of part of the Caldmodulin gene (CMD) was performed using the primers CMD5 (CCGAGTACAAGGARGCCTTC) and CMD6 (CCGATRGAGGTCATRACGTGG). These fragments were sequenced with the ABI Prism® Big DyeTM Terminator v.3.0 Ready Reaction Cycle sequencing Kit. Samples were analysed on an ABI PRISM 3700 Genetic Analyzer and contigs were assembled using the forward and reverse sequences with the programme SeqMan from the LaserGene package. A multigene analysis was carried out by comparing part of the β-tubulin gene and part of the Caldmodulin gene sequences on GenBank and on http://www.cbs.knaw.nl/.

### 9. Results

Bioinformatic analysis of the sequence data (partial fragment BT and partial fragment CMD) is included in Appendix 1. The results of the two-gene sequence analysis show a 100% match with *A. luchuensis.*

Results of mycotoxin analysis are included in Table 1.

| **Table 1. Results from mycotoxin analysis** | |
|---|---|
| **Mycotoxin:** | **FL100SC** |
| Alternariol methyl ether | Absent (*) |
| Alternariol | Absent (*) |
| Aflatoxin B1 | Absent (*) |
| Aflatoxin B2 | Absent (*) |
| Aflatoxin G1 | Absent (*) |
| Aflatoxin G2 | Absent (*) |
| 3-Ac-DON | Absent (*) |
| Deoxynivalenol (DON) / Vomitoxin | Absent (*) |
| Diacetoxyscirpenol (DAS) | Absent (*) |
| Fumonisin B1 | Absent (*) |
| HT-2 Toxin | Absent (*) |
| Ochratoxin A (OTA) | Absent (*) |
| Sterigmatocystin | Absent (*) |
| T-2 Toxin | Absent (*) |
| a+ß Zearalenol (ZEL) | Absent (*) |
| Zearalenone (ZEA) | Absent (*) |
| Fumonisin B2 | Absent (*) |
| CPA, Cyclopiazonic acid | Absent (*) |
| Roquefortine C | Absent (*) |
| Citrinin | Absent (*) |
| Mycophenolic acid | Absent (*) |
| Patulin | Absent (*) |
| Penitrem A | Absent (*) |
| Penicillic acid | Absent (*) |

*: Manualy entered results

## 10. Conclusion

Analysis of the sequence data (partial fragment BT and partial fragment CMD) showed that the FL100SC strain shared sequences with isolates of *Aspergillus luchuensis*.

No known mycotoxins were detected in the analysed extract of FL100SC *Aspergillus luchuensis.*

The name of *Aspergillus acidus* (previously known as *Aspergillus foetidus* var. *pallidus* and *Aspergillus foetidus* var. *acidus*) has recently been changed to *Aspergillus luchuensis*. *Aspergillus acidus*, *Aspergillus foetidus* var. *pallidus*, *Aspergillus foetidus* var. *acidus* are synonyms of *Aspergillus luchuensis*.

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## 12. Appendices

*Appendix 1: Bioinformatic multigene analysis results*
*Appendix 2: Curriculum vitae of study director*

## Appendix 1: Bioinformatic multigene analysis results

FL100SC part of the β-tubulin gene

CCGGCCAGTGTGTAAGTGCCAATATGTTCTTCGAATGATTGCCCCCCCGGGTTCTTGATTGGTGTTCGG
TGGACTAAACAACAAATGATGGTGGTTAGGGTAACCAAATTGGTGCTGCTTTCTGGTACGTATTCACTG
CCACTGGATTGGGGATGGATAACATCATCTCTCAAGCTATCTCGGCTTGAGTTCAGATGTTATTTATCG
GGTATATAGCTATCGGGTTAAGAACACGTCTAACAACTCAACAGGCAGACCATCTCTGGCGAGCACGG
CCTTGACGGCTCCGGTGTGTAAGTGCAACTTTTTCACACCTCTCAATTGGTCGACAATGTGGAAAGGAT
TGGGTTTCCTGACACGCAGGATAGTTACAATGGCACCTCCGACCTCCAGCTGGAGCGCATGAACGTCT
ACTTCAACGAGGTTAGATCACACCGTCCCTGAGTTTTTTCACGACAATATCATCAATGTCCTGACCACT
TCAGCAGGCTAGCGGTAACAAGTATGTCCCCCGTGCCGTCCTCGTCGATCTCGAGCCCGGTACCATGGA
CGCTGTCCGTGCCGGTCCCTTCGGCCAGCTCTTCCGTCCCGACAACTTCGTCTTCGGCCAGTCCGGTGC
TGGTAACAACTGGGCCAAGGG

FL100SC part of the Calmodulin gene

TCTCCCTCTTTGTGAGTGCTCCCTGAATAAACCCCCGATCACTCAAATTGATGACCTATCATGACCGGC
TCATAATGCTAATGTATTCTCGAACTCAATAGGACAAGGATGGCGATGGTGGGTGGAATTCTGTCCCCT
TTACGTTTTACCCGTAGCGCCCGATCCGACCGCGGGATTTCGACAGCCATTTCCCCATCGATCTGAATC
ATTATACTGATGTAATCTGGAAATAGGCCAGATCACCACCAAGGAGCTCGGCACTGTGATGCGCTCCC
TCGGCCAGAACCCCTCCGAGTCTGAGCTTCAGGACATGATCAACGAGGTTGACGCTGACAACAACGGA
ACGATCGACTTCCCCGGTATGTGATAGATCTACGCCTGTAGGGCGGGAATGCCGTATGGGTTGTGATT
GACTTTTGCCGCCAGAATTCCTTACCATGATGGCTCGTAAGATGAAGGACACCGACTCCGAGGAGGAA
ATCCGCGAGGCTTTCAAGGTCTTCGACCGCGACAACAATGGTTTCATCTCCGCCGCGGAGT

**Appendix 2: Curriculum vitae of study director**

*Name*: Robert A. Samson

*Curriculum vitae:*

- Since 1970 employed by the Royal Netherlands Academy of Science (Amsterdam) at the CBS-KNAW Fungal Biodiversity Centre and groupleader of the Applied and Industrial Mycology.
- Adjunct Professor in Plant Pathology of the Faculty of Agriculture, Kasetsart University Bangkok, Thailand since July 15, 2002.
- Since January 2009 visiting professor at Instituto de TecnologiaQuimica e Biologica of the Universidade Nova de Lisboa in Portugal.
- Honorary Doctor of Agricultural Sciences of the Faculty of Natural Resources and Agricultural Sciences at the Swedish University of Agricultural Sciences in Uppsala (October 3 2009).

*Specialization:*
Main field: Systematic mycology of *Penicillium* and *Aspergillus*, food-borne fungi.
Other fields: Mycoflora of indoor environments, entomopathogenic, thermophilic fungi, scanning electronmicroscopy.
Current research interests:

- Taxonomy of *Penicillium* and *Aspergillus*
- Food-borne fungi with emphasis on heat resistant and xerophilicmoulds
- Moulds in indoor environments
- Entomogenous fungi

*Honours, Awards, Fellowships, Membership of Professional Societies:*

- Secretary General of the International Union of Microbiological Societies (IUMS)
- Member of the Executive Board of the International Union of Microbiological Societies since 1986
- Chairman of the IUMS International Commission on *Penicillium* and *Aspergillus*
- Vice Chairman and member of the IUMS International Commission on Food Mycology
- Member of the International Commission of the Taxonomy of Fungi
- Chairman of the IUMS International Commission on Indoor Fungi
- Honorary Member of the American Mycological Society
- Honorary Member of the Hungarian Society of Microbiology

**Centraalbureau voor Schimmelcultures**
Fungal Biodiversity Centre
*Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW)*

# Report

# Identification of one *Aspergillus* strain and the analysis of secondary metabolites

**In order of:** M/s Advanced Enzyme Technologies LTd
A 61/62 Malegaon MIDC,
Sinnar Nashik-422113, India

**Contact:** Dr. Anil Kumar Gupta
Sr. Manager R & D

**Our ref.:** TM 09.085

**Your ref.:** e-mail dd. July 7 2009

**Analyses of:** ASNSC *Aspergillus niger*

# Centraalbureau voor Schimmelcultures

Fungal Biodiversity Centre

*Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW)*


**Aim of the investigation**

Fungi are known to produce many secondary metabolites. Some of these metabolites are considered to be mycotoxins. The aim of this investigation was to analyse one fungal isolate for toxic metabolites. Cultivation and extracting of the strain was performed at the CBS Fungal Biodiversity Centre in Utrecht. The extracts were sent to Prof. Jens C. Frisvad (University of Denmark, Lyngby), who analysed the extract on the presence of metabolites and compared them with all important toxins which may be significant.

**Methods**

*Metabolite analyses*

The following sample was examined:

**ASNSC *Aspergillus niger***

The strain was cultured on the following media which are given the most optimal expression for the production of secondary metabolites: Czapek yeast autolysate agar (CYA), Malt Extract Agar (MEA) and yeast extract sucrose agar (YES). (Frisvad and Filtenborg, 1989; Frisvad and Thrane, 1993; Frisvad et al, 2007). The isolate was incubated for 7 and 14 days in darkness at 25°C. For extrolite analysis three agar plugs with fungal colonies of each plate were combined and extracted using extraction solvent dichlormethane:methanol:ethylacetate (2:1:3) with 0.5% formic acid was used (Smedsgaard, 1997). The samples were subsequently analysed by high performance liquid chromatography (HPLC) with diode array detection (DAD) and Time-of-flight mass spectrometry (TOF-MS) (Frisvad and Thrane, 1993, Nielsen and Smedsgaard, 2003). The metabolites found were compared to a library made from authentic standards run at the same conditions.

*Identification*

DNA was extracted from the mycelium using the Mo-Bio laboratories; UltraClean Microbial DNA Isolation Kit. Fragments containing a part of the β-tubulin gene were amplified with Polymerase Chain Reaction (PCR) using the primers Bt2a (GGTAACCAAATCGGTGCTGCTTTC) and Bt2b (ACCCTCAGTGTAGTGACCCTTGGC). This fragment was sequenced with the ABI Prism® Big DyeTM Terminator v.3.0 Ready Reaction Cycle sequencing Kit. Samples were analysed on an ABI PRISM 3700 Genetic Analyzer and contigs were assembled using the forward and reverse sequences with the programme SeqMan from the LaserGene package.

**Results and conclusion**

Phenotypic analysis of the culture showed black colored biseriate conidial heads, and typical *Aspergillus* conidiophores, indicating that this strain is a member of *Aspergillus* section *Nigri*. Analyses of the sequence data (Bt2a-Bt2b region) showed that the isolate ASNSC *A. niger* shared sequences with the type culture of *Aspergillus foetidus*.

No known mycotoxins were found in the extracts of this culture. The results of the HPLC analyses are summarized in appendix 1.

**Centraalbureau voor Schimmelcultures**
Fungal Biodiversity Centre
*Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW)*

**References**

Frisvad, J.C. & Filtenborg, O., 1989. Terverticillate Penicillia: chemotaxonomy and mycotoxin production. Mycologia 81: 837-861

Frisvad, J.C. & Thrane, U. 1993. Liquid column chromatography of mycotoxins. In: Betina, V. (ed.): Chromatography of mycotoxins. Techniques and applications. Journal of Chromatography Library 54: 253-372. Elsevier, Amsterdam.

Frisvad, J.C., Smedsgaard, J., Samson, R., Larsen, T. and Thrane, U. 2007. Fumonisin B2 production by *Aspergillus niger*. Journal of agricultural and food chemistry 55 (23): 9727-9732

Nielsen, K.F. and Smedsgaard, J. Fungal metabolite screening: database of 474 mycotoxins and fungal metabolites for dereplication by standardised liquid chromatography-uv-mass spectrometry methodology, Journal of Chromatography, A 1002 (2003), pp. 111–136

Samson, R.A., Hoekstra, E.S. and Frisvad, J.C. 2004. Introduction to food- and airborne fungi, seventh edition, Centraalbureau voor Schimmelcultures, the Netherlands.

Smedsgaard, J. (1997). Micro scale extraction procedure for standardized screening of fungal metabolite production in culture. Journal of Chromatography A 760: 264-270.

Utrecht, November 4 2009

(b) (6)

Dr. R.A. Samson

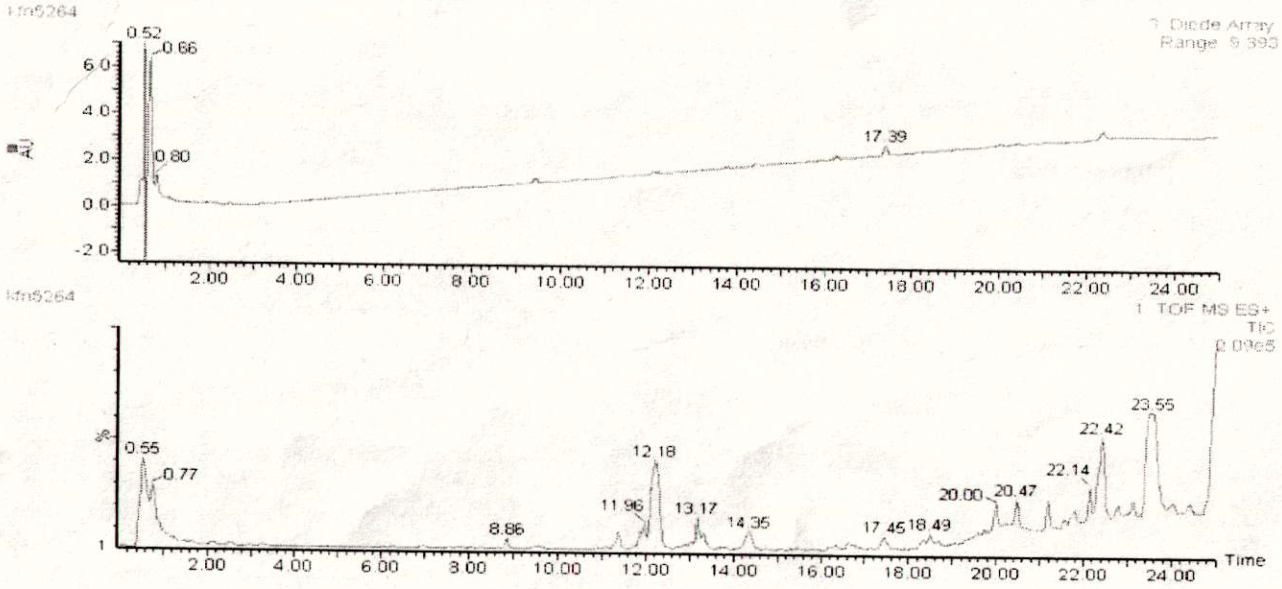
(b) (6)

Ing J. Houbraken

**Centraalbureau voor Schimmelcultures**
Fungal Biodiversity Centre
*Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW)*

Appendix 1. Overview HPLC spectra

TM09.085-02 *Aspergillus foetidus*

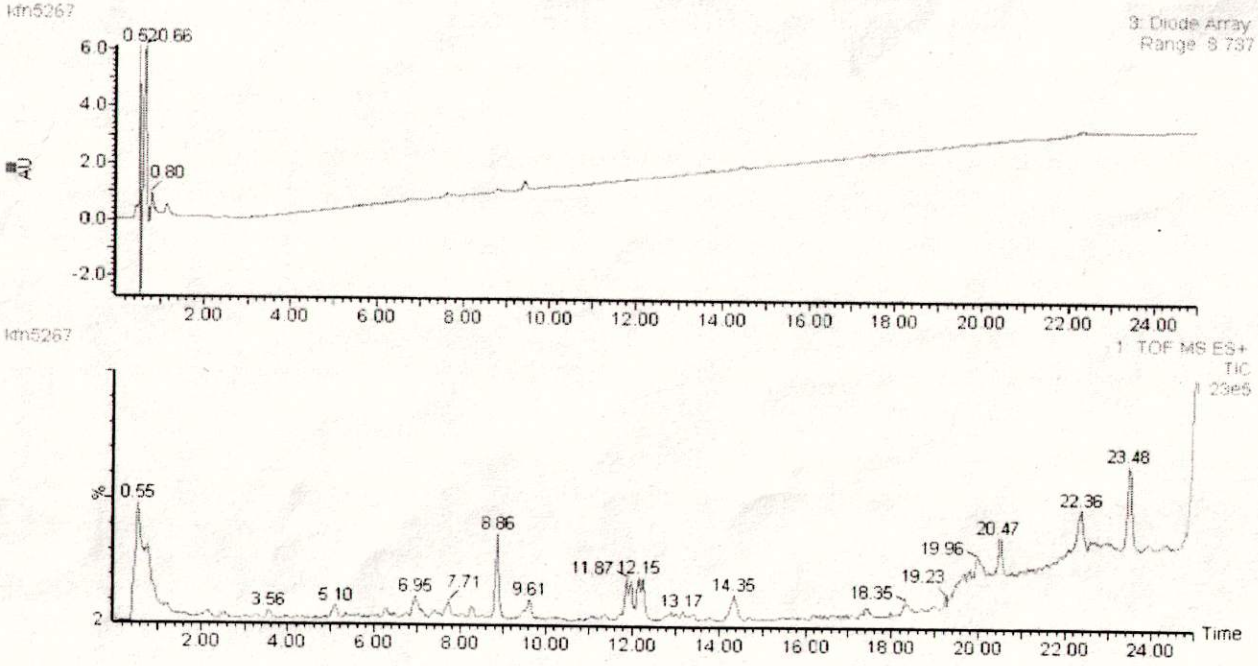


# Centraalbureau voor Schimmelcultures

Fungal Biodiversity Centre

*Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW)*

TM09.085-05 *Aspergillus foetidus*



# CABI IDENTIFICATION REPORT



**Our ref**: YI2/13/H64

**Your ref**: Quote IDQ/03/13

**Reporting to:**

Dr A.K. Gupta
Advanced Enzyme Technologies Ltd.
Plot No. A-61/62
Malegaon
Tal. Sinnar, Dist. Nashik
422113
India

**Date**: 30 July 2013

**CONFIDENTIAL**

**Enquiry YI2/13/H64 Final Identification Report (2)**

**Date received: 08/04/2013** **Date started: 09/04/2013** **Date completed: 30/07/2013**

**Description of material received:**

The customer submitted 1 sample for microbial identification.

A unique CABI reference number (IMI number) was assigned to each of the customer's samples. Details of the samples received and the customer's requirements are listed below.

| Customer sample | IMI Number | Description | Processing requirement | Service level |
|---|---|---|---|---|
| ASNSC | 502637 | Microbial culture | Identification by dual locus sequencing with Full report | Normal |

**Methods:**

IMI 502637 was processed using ITS and partial calmodulin rDNA sequencing analysis.

All procedures were validated and processing undertaken in accordance with CABI's in-house methods.

Procedures involved the following steps:

The original samples were subjected to a purity check.

www.cabi.org

Our ref.: YI2/13/H64

Your ref.: Quote IDQ/03/13

Dr A.K. Gupta
Advanced Enzyme Technologies Ltd.
Plot No. A-61/62
Malegaon
Tal. Sinnar, Dist. Nashik
422113
India

Date: 30 July 2013

Dear Dr Gupta

**Confidential**

**Enquiry YI2/13/H64 Final Identification Report (2)**

In reply to your enquiry received on 08 April 2013, and with reference to our previous reports of 3rd May and 31st May 2013, I am pleased to provide our Final Identification Report on the material you submitted.

Yours sincerely

(b) (6)

**Miss T.S. Caine**
ID Operations Manager
Microbial Identification Service
t.caine@cabi.org

Molecular assays were carried out on the samples using nucleic acid as a template. A proprietary formulation [microLYSIS®-PLUS (MLP), Microzone, UK)] was subjected to the rapid heating and cooling of a thermal cycler, to lyse cells and release deoxyribonucleic acid (DNA).

Following DNA extraction, Polymerase Chain Reaction (PCR) was employed to amplify copies of the rDNA in vitro.

The quality of the PCR product was assessed by undertaking gel electrophoresis.

PCR purification step was carried out to remove unutilised dNTPs, primers, polymerase and other PCR mixture compounds and obtain a highly purified DNA template for sequencing. This procedure also allowed concentration of low yield amplicons.

Sequencing reactions were undertaken using BigDye® Terminator v3.1 kit from Applied Biosystems (Life Technologies, UK) which utilises fluorescent labelling of the chain terminator ddNTPs, to permit sequencing.

Removal of excess unincorporated dye terminators was carried out to ensure a problem-free electrophoresis of fluorescently labelled sequencing reaction products on the capillary array AB 3130 Genetic Analyzer (DS1) DyeEx™ 2.0 (Qiagen, UK) modules containing prehydrated gel-filtration resin were optimized for clean-up of sequencing reactions containing BigDye® terminators. Dye removal was followed by suspension of the purified products in highly deionised formamide Hi-Di™ (Life Technologies, UK) to prevent rapid sample evaporation and secondary structure formation.

The samples were loaded onto the AB 3130 Genetic Analyzer and sequencing undertaken to determine the order of the nucleotide bases, adenine, guanine, cytosine, and thymine in the DNA oligonucleotide.

**Results:**

| Customer sample | IMI Number | Identification and comments |
|---|---|---|
| ASNSC | 502637 | **Identified as: Aspergillus niger agg.**<br><br>**Process:** This isolate was processed using ITS and partial calmodulin rDNA sequencing and identified from the results of calmodulin sequencing using the BLAST algorithm with the NCBI GenBank sequence database.<br><br>**Result:** The calmodulin sequence obtained from this sample showed top matches at 100% identity to members of the *Aspergillus niger* species aggregate, including published sequences from reference culture collections [e.g. 100% to published *A. tubingensis*: EF661152 (NRRL 4750 ) cited in Peterson S.W. *et al.* (2008). Phylogenetic analysis of *Aspergillus* species using DNA sequences from four loci. *Mycologia* **100**(2): 205-226. Also 100% to published *A. coreanus*: FJ491702 (CBS 119883 ) cited in Varga J. *et al.* (2011). New and revisited species in *Aspergillus* section Nigri. *Stud. Mycol.* **69**(1): 1-17. The ITS sequence obtained from this sample also showed 100% matches to multiple ITS sequences from members of the *Aspergillus niger* aggregate. Cluster analysis undertaken on the results from calmodulin sequencing, showed that this sample belongs to a group that is close to but distinct from *Aspergillus tubingensis*.<br><br>**Comment:** Members of the *Aspergillus niger* species aggregate are common throughout the world and frequently isolated from many |

| Customer sample | IMI Number | Identification and comments |
|---|---|---|
| | | different substrata including soil, plants, food, water, air etc. They have been reported to produce a wide range of mycotoxins. All members of this group are nevertheless classed in ACDP hazard group 1.<br><br>**Destination:** This material will be discarded. |

**Opinions and Interpretations:**

Where matches of 99-100% identity are obtained, identification is provided to species level, or where appropriate, to species aggregate (as in the case of IMI 502637), provided that matches include a sequence derived from type or other validated culture and when there is a clear sequence distinction between taxa.

**Authorisation:**

I certify that this report has been checked and approved.

Signed:

(b) (6)

Miss T.S. Caine
ID Operations Manager
Microbial Identification Service
t.caine@cabi.org

# ANNEX I

## 1 TITLE: ALLERGENICITY ASSESSMENT – RHIZOPUS ORYZAE LIPASE PRODUCED BY GMM ASPERGILLUS NIGER (STRAIN FL100SC)

M/s. Advanced Enzyme Technologies Ltd.
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Tel. no: +91 22 41703200, Fax no: +91 22 25835159
Email: info@advancedenzymes.com

## TABLE OF CONTENTS

## 2 OBJECTIVE

To assess the allergenicity of *Rhizopus oryzae* lipase produced by genetically modified

*Aspergillus niger* (strain FL100SC)

## 3 INTRODUCTION

Allergenicity is the potential of a substance (e.g. food or food components such as proteins) to cause an allergy. Food allergy is an adverse reaction to food and represents an important public health problem. In allergic individuals, sometimes, minute amounts of a food that is well tolerated by the vast majority of the population can cause serious symptoms and death. It is not the allergen *per se*, but the allergic person's abnormal reaction to the allergen that causes the adverse health effect. At present, there is no single definitive test that can be relied upon to predict allergic responses in humans to newly expressed protein.

In order to address the allergenicity of enzymes by oral route in consumers, Bindslev et al (2006) assessed the possible clinical sensitizing ability of 19 enzymes. The investigation comprised enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and protein engineered variants. The study comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. Further, active forms of the enzymes were tested, i.e. before the enzymes were denatured / inactivated owing to heat, pH changes, etc., in the final commercial product. This aspect added weightage to the safety findings of the study, which concluded that ingestion of food enzymes in general is not likely to be a concern with regard to food allergy.

In a separate analysis conducted earlier by the AMFEP Working Group on Consumer Allergy Risk from Enzyme Residues in Food by Dauvrin et al (1998), it was concluded that based on the existing literature and from a scientific point of view there was no indication that enzyme residues in foods may represent an unacceptable risk to consumers. The analysis also indicated that even when high daily doses of enzymes are ingested as digestive aids, there have not been any reports on gastrointestinal allergy to enzymes, after many years of daily intake.

However, over the last decade, bioinformatics methods have been widely used for collecting, storing, and analysing molecular and/or clinical information of importance for allergy. Information obtained from bioinformatics, coupled with experimental data, wherever necessary, is the approach postulated by the joint Food and Agriculture Organisation and World Health Organisation (FAO/WHO) Expert Consultation on Allergenicity of Foods Derived from Biotechnology, to address this point. This approach takes into consideration the evidence derived from several types of information and data since no single criterion is sufficiently predictive.1

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

[1] Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology;22 – 25 January 2001; 1-29 | http://www.fao.org/ag/agn/food/pdf/allergygm.pdf

sequence of the expressed protein, using a window of 80 amino acids and a suitable gap penalty, or (b) identity of 6 contiguous amino acids.

It is pertinent to note that the identical peptide match method using a peptide length of six amino acids has attracted much criticism, since it generates too many false positives in testing of potential allergenicity. Further, Stadler et al, 2003, have shown that the FAO/WHO method, on an average, yields only about 1 true allergen for about every 200 proteins that are predicted to be allergenic. In 2006 and 2009, the European Food Safety Authority (EFSA) released a guidance which is in line with the recommendations of the Codex Alimentarius as regards the assessment of the allergenicity of GM foods and in which improved in silico testing for prediction of potential allergenicity is recommended. The 'Scientific Opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed: EFSA Panel on Genetically Modified Organisms (GMO Panel)', in one of its conclusions states: [2]

> *Methods searching a complete identity over 6 contiguous amino acids to known allergens are associated with very poor specificity (many false positives). Therefore, it is in general not considered appropriate.*

Hence, in the present study, results covering 35% identity in the amino acid sequence of the expressed protein, using a window of 80 amino acids and a suitable gap penalty are presented. Further, results obtained for sequence alignments covering exact match for 8 contiguous amino acids (Codex Alimentarius, 2003 & 2008) are also presented.[3]

As recommended in the EFSA draft scientific opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed and by other bioinformatics expert groups, default settings for the substitution matrix and gap penalties were employed for conducting the sequence homology searches.[4,5]

## 4 METHODOLOGY

### 4.1 BIOINFORMATICS SEARCHES

In order to assess the allergenicity of the protein under scrutiny, sequence specific homology searches were conducted in the Food Allergy Research and Resource Program (FARRP),

---

[2] EFSA Panel on Genetically Modified Organisms (GMO); Draft Scientific Opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed. EFSA Journal 2010; 8(7):1700. [168 pp.]. | http://www.efsa.europa.eu/en/efsajournal/doc/1700.pdf

[3] Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants - CAC/GL 45-2003 | http://www.fao.org/fileadmin/user_upload/gmfp/docs/CAC.GL_45_2003.pdf

[4] EFSA Panel on Genetically Modified Organisms (GMO); Draft Scientific Opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed. EFSA Journal 2010; 8(7):1700. [168 pp.]. | http://www.efsa.europa.eu/en/efsajournal/doc/1700.pdf

[5] Allermatch™, a webtool for the prediction of potential allergenicity according to current FAO/WHO Codex alimentarius guidelines; BMC Bioinformatics 2004, 5:133 | http://www.biomedcentral.com/content/pdf/1471-2105-5-133.pdf

commonly known as 'Allergen Online' database, and hosted by University of Nebraska – Lincoln. [6]

### 4.1.1 FARRP Database

The FARRP (AllergenOnline) database is intended for use as a tool for evaluating the safety of proteins included in foods through processing or genetic modification. A key component in the evaluation process is comparison of candidate products (proteins) with those of known allergens using a bioinformatics approach such as FASTA. It is therefore important to have scientific evidence that the database entries are allergens or probable (putative) allergens in order to maximize the reliability of bioinformatics searches.

In addition to the full-length FASTA search, the FARRP database has options to automatically scan an 80 amino acid window, looking for matches of at least 35% identity, and to scan for shorter identical segments of 8 contiguous amino acids.

The FARRP database is built around the FASTA package, version 35.04. The scoring matrix used on the AllergenOnline website is a BLOSUM 50 that is weighted to favor identical matches between amino acids that are likely to significantly impact the overall protein structure, with less weight given to those unlikely to significantly impact the structure, and less weight given to "similar" amino acids.

Homology searches were conducted on the test / query sequence, employing the following three criteria available on FARRP database:

1. FASTA alignments for an 80 amino acids sliding window (35% sequence identity cutoff used for the 80 amino acids sliding window alignments - FAO/WHO Guidelines, 2001 & Goodman et al, 2008. [7, 8]

   The percentage identity (PID) was determined in order to assess whether the test / query sequence shows >35% identity to known allergens using a sliding window of 80 amino acids.

2. % sequence identity by Full FASTA alignment (should be >50% overall identity) - Goodman et al, 2008. [9]

3. Exact match for 8 contiguous amino acids – Codex Alimentarius, 2003 & 2008. [10]

## 4.2 PEPSIN-DIGESTION ASSAY

A general consensus has been reached that proteins susceptible to gastrointestinal digestion are inherently safer than those that are stable with respect to allergenicity.[11]

---

[6] FARRP / AllergenOnline | http://www.allergenonline.org/databasefasta.shtml
[7] Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology;22 – 25 January 2001; 1-29 | http://www.fao.org/ag/agn/food/pdf/allergygm.pdf
[8] Goodman RE1, Vieths S, Sampson HA, Hill D, Ebisawa M, Taylor SL, van Ree R.; Allergenicity assessment of genetically modified crops--what makes sense?; Nat Biotechnol. 2008; 26(1):73-81 | http://www.ncbi.nlm.nih.gov/pubmed/18183024
[9] *Ibid. 7*
[10] Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants - CAC/GL 45-2003 | http://www.fao.org/fileadmin/user_upload/gmfp/docs/CAC.GL_45_2003.pdf

With the above understanding as the basis, pepsin digestion test was carried on the test enzyme protein, *Rhizopus oryzae* lipase from *Aspergillus niger* (strain FL100SC).

The protocol used for this assay is based on the detailed study undertaken by Thomas *et al*, 2004, which takes into consideration the guidelines provided by the Codex Alimentarius Commission, 2003. The same study has been referred to in the EFSA Journal (Reference: EFSA Journal 2010; 8 (7):1700. Annex 4. "Assessment of allerginicity of newly expressed) proteins in GMOs using *in vitro* and cell–based tests".

Briefly, the assay is performed under standard conditions of 10 units of pepsin activity per microgram of test protein. The FAO/WHO in 2001 recommended using pH 2.0 for the assay. However, a multi-laboratory evaluation of the assay protocol conducted in 2004 by Thomas *et al.* showed that there is more consistency between laboratories in detection of protein fragments at pH 1.2, rather than at pH 2.0. Further, the multi-laboratory evaluation of the assay protocol indicated that there was no appreciable difference in the time to disappearance of the full-length protein or protein fragments at pH 1.2 or 2.0. This is in agreement with historical data as well as US Pharmacopoiea recommendations. Hence, the pepsin digestion assay was conducted at pH 1.2. The pepsin digestion reaction was performed at 37°C. Samples were removed at specific times and the activity of pepsin was quenched by neutralization with a carbonate buffer containing the SDS-PAGE sample loading buffer and a reducing reagent. The timed digestion samples were separated by SDS-PAGE and stained with Coomassie to evaluate the extent of digestion. Following destaining of the gels, the gels were assessed for the final time point for which the full length protein or fragment was detectable. [12, 13]

Pepsin digestion assay shows that Lipase from *Aspergillus niger* agg. (strain FL100SC) 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.

[11] Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology; 22 – 25 January 2001; Topic 5: Stability of Known Allergens (Digestive and Heat Stability) | http://www.fao.org/ag/agn/food/pdf/bi07al.pdf

[12] Codex Alimentarius (2003). Codex Alinorm 03/34: Joint FAO/WHO Food Standard Programme, Codex Alimentarius Commission, Twenty-Fifth Session, Rome, Italy 30 June-5 July, 2003. Appendix III; http://www.biosafety.be/ARGMO/Documents/codex.pdf .

[13] Thomas K, Aalbers M, Bannon GA, Bartels M, et al.; 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 (2004); 87–98.

# 5 RESULTS

## 5.1 BIOINFORMATICS SEARCHES

A snapshot of the results obtained following homology sequence searches in FARRP database is presented in the table below.

| | | | | |
|---|---|---|---|---|
| ***Rhizopus oryzae* lipase** | - | - | - | - |

As is clear from the information presented in the table:

### *5.1.1.1 In case of an 80 amino acids sliding window*

No match with any known allergen was observed in case of *Rhizopus oryzae* lipase, enzyme protein in FARRP database.

### *5.1.1.2 In case of a full length FASTA alignment*

No match with any known allergen was observed in case of *Rhizopus oryzae* lipase, enzyme protein in FARRP database.

### *5.1.1.3 In case of peptide match of complete identity over 8 contiguous amino acids*

No match with any known allergen was observed in case of *Rhizopus oryzae* lipase, enzyme protein in FARRP database.

Pertinent to note is that no match with any known allergen was observed when complete identity over 8 contiguous amino acids, 35% identity over 80 amino acid sliding window and 50% identity on a full length basis was tested.

## 6 CONCLUSION

Bioinformatics searches show that *Rhizopus oryzae* lipase shares no homology to known allergen over a sliding window of 80 amino acids or full length. Additionally, no hits were obtained for a match of 8 contiguous amino acids.

In a separate analysis conducted earlier by the AMFEP Working Group on Consumer Allergy Risk from Enzyme Residues in Food by Dauvrin et al (1998), it was concluded that there was no indication that enzyme residues in foods may represent an unacceptable risk to consumers. The analysis also indicated that even when high daily doses of enzymes are ingested as digestive aids, there have not been any reports on gastrointestinal allergy to enzymes, after many years of daily intake.

It is worthwhile noting that:

(i) food enzymes have a long history of safe use in food, with no indication of adverse effects or reactions,

(ii) a wide variety of enzyme classes are naturally present in food

(iii) the enzyme is typically used in small amount during food processing, and owing to unconducive pH & temperature conditions, substrate depletion, etc. is unable to perform its technological function in the final food

(iv) any residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system.

In the light of the above points, ingestion of a food enzyme *Rhizopus oryzae* lipase is not a concern in terms of food allergy.

**ANNEX J**

**1. Title: Report on assessment of the leakage of active enzyme and / or immobilization support materials / resins into food**

**Unique Study Code: ANRD/AET/FL/003**

**Advanced Enzyme Technologies Ltd.**

Date: January 2015

| (b) (6) | (b) (6) |
|---|---|
| **Study Director** | **Study Monitor** |
| Mr. Piyush Rathi<br>Advanced Enzyme Technologies Ltd<br>Email: *pcrathi@advancedenzymes.com*<br>*http://www.advancedenzymes.com* | **Dr. Anuradha Chitnis**<br>Advanced Enzyme Technologies Ltd<br>Email: *anuradha@advancedenzymes.com*<br>*http://www.advancedenzymes.com* |

*2. Table of Contents*

*3. Summary*

An immobilized preparation of lipase from *Rhizopus oryzae*, var. *delamar* was assessed for leakage of the immobilized enzyme into food.

Studies showed absence of protein in the final food, which was ascertained by determining the nitrogen content in the final food.

*4. Quality Statement*

This study is carried out in compliance with current quality standards for EU food additive applications.

Date:

| | |
|---|---|
| (b) (6) | (b) (6) |

Name: Mr. Piyush Rathi
Study Director

Name Dr. Anuradha Chitnis
Study Monitor

*5. Introduction*

The safety of the enzyme has been already addressed in our dossier for Lipase from *Rhizopus oryzae, var. delamar.* However, the data included in the present report aims to provide more information regarding the enzyme system and the potential presence of enzyme residues in oil/fat modified with the immobilized enzyme.

The immobilization support/carrier material used in manufacture of the enzyme product complies with the regulations of the Council of Europe Committee of Ministers' Resolution ResAP(2004)[1] on ion exchange and adsorbent resins used in the processing of foodstuffs.
In this evaluation, a model reaction was conducted using oil/fat as substrate and the immobilized lipase as the catalyst. The modified oil/fat was then analyzed for a change in nitrogen content as an indicator for protein leaching.

*6. Study Title and Unique Study Code*

Report on assessment of the leakage of active enzyme and / or immobilization support materials / resins into food.

Unique Study Code: ANRD/AET/FL/003

*7. Study Objective*

The objective of the study was to assess the leakage of of active enzyme and / or immobilization support materials / resins into final food.

*8. Study Location*

Advanced Enzyme Technologies Ltd.

*9. Dates of the Study*

5/01/2015-6/01/2015

*10. Details of the Enzyme Sample Used*

| **Table 1.Details of test product** | | |
|---|---|---|
| Product name | Manufacturer | Lot No., Manufacture Date |
| ADDZYME RD 165G | Advanced Enzyme Technologies Ltd. | Lot number: 111478-T<br>Manufactured: Nov 2014<br>Expiry:* |
| * Product will lose not more than 10% activity in 12 months from the date of manufacturing, if stored at 5°C. | | |

[1] http://www.contactalimentaire.com/fileadmin/ImageFichier_Archive/contact_alimentaire/Fichiers_Documents/Resolution_anglais/resolution_2004-3_ang.pdf

*11. Materials and Methods*

***Leaching of enzyme into final food***

A model interesterification reaction was carried out with the enzyme.

Interesterification was carried out using 3.75g Palm Stearin, 11.25g Coconut oil and 45 ml n-Hexane. The mixture was equilibrated at 55°C to enable complete dissolution of the substrates. Suitable sample was withdrawn. The reaction was initiated by 1.5g of the immobilized enzyme and continued at 55°C in shaker cum incubator at 200 rpm for 4hours. The enzyme was filtered off after the reaction. The samples, before the addition of enzyme and 4 hours post the reaction were subjected to solvent evaporation in a rotary evaporator. Thus obtained fat/oil samples were analysed for nitrogen/ protein content. All analyses were carried out in duplicate and averaged.

The nitrogen contents of the substrate and the reaction mass after 4 hours of reaction were analysed by using semi-automated Kjeldahl equipment for digestion and distillation, followed by manual titration. The nitrogen content was converted to protein content using 6.25 as conversion factor.

***Leaching of immobilization support/resin into final food***

It is pertinent to note that the immobilization support/carrier material complies with the regulations of the Council of Europe Committee of Ministers' Resolution ResAP(2004)[2] on ion exchange and adsorbent resins used in the processing of foodstuffs.

**Test product**:

1. Product name – Addzyme RD 165 G
   B.No – 111478-T
   Date of manufacture – 10/11/2014
   Manufacturer – Advanced Enzymes Technologies Ltd

[2] http://www.contactalimentaire.com/fileadmin/ImageFichier_Archive/contact_alimentaire/Fichiers_Documents/Resolution_anglais/resolution_2004-3_ang.pdf

*12. Results*

***Leaching of enzyme into final food***

| Samples | Nitrogen % | Protein content (%) |
|---|---|---|
| Initial reaction mix (Before enzyme addition) | 0.0342 | 0.214 |
| ADDZYME RD 165G (10% Enzyme loading w.r.t substrate) | 0.0136 | 0.085 |
| Egg white powder (Reference Standard) | 13.651 | 85.32 |

As shown in the table, the final food sample did not show any increase in the amount of nitrogen content, which was reflected in a comparative value for the protein content calculated based on the nitrogen content. This is suggestive if the fact that the enzyme sample, which is basically proteinaceous in nature, does not leach into food.

***Leaching of immobilization support/resin into final food***

Importantly, the use of the immobilization support/carrier material in food applications is strictly limited to the applications, restrictions, and operating requirements that are described in Resolution ResAP(2004)[3] on ion exchange and adsorbent resins used in the processing of foodstuffs.

*13. Discussion*

The initial reaction mix shows the presence of nitrogen. This might be as a result presence of non-protein nitrogen, as the oil/fat is not expected to contain protein. Post reaction samples show a reduced nitrogen/protein content, indicating that the enzyme has not leached into the oil.

Additionally, the immobilization support/carrier material complies with Resolution ResAP (2004)[4] on ion exchange and adsorbent resins used in the processing of foodstuffs. This in itself indicates that, the immobilization support/carrier, *inter alia*, does not transfer its constituents to foodstuffs in quantities which could endanger human health or bring about an unacceptable change in the composition of the foodstuffs or deterioration in the organoleptic characteristics thereof.

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3 http://www.contactalimentaire.com/fileadmin/ImageFichier_Archive/contact_alimentaire/Fichiers_Documents/Resolution_anglais/resolution_2004-3_ang.pdf

4 *Ibid.*

*14. Conclusion*

It can be concluded from the result that since there has been no increase in the nitrogen/protein content of the reaction mass after addition and reaction with the said immobilized enzyme preparations, there is no leaching of the enzyme from the absorbent to the oil/fat, under the conditions of the study.

*15. List of Appendices*

*Appendix 1 – Curriculum Vitae of the Study Director & Study Monitor*

*Appendix 2 – Certificate of Analysis of the product used for the study*

*Appendix 3 – List of Reagents*

*Appendix 4 – List of Instruments*

*Appendix 5 – Procedure for Immobilization*

*Appendix 6 – Procedure for reaction*

*Appendix 7 – Procedure for nitrogen/ protein estimation.*

Two pages of Curriculum Vitae removed in accordance with the Privacy Act of 1974.

## Appendix 1 - Certificate of Analysis of the product used for the study

advanced enzymes
Where ENZYME is Life

**Advanced Enzyme Technologies Ltd.,**
Plot No. A-61/62, MIDC, Malegaon, Tal. Sinnar,
Dist. Nashik - 422 113, Maharashtra, India.
Tel.: +91-2551-230 043 / +91-2551-230 044, Fax: +91-2551-230 816
Email: info@enzymeindia.com, Web.: www.advancedenzymes.com

### QUALITY ASSURANCE DEPARTMENT

### CERTIFICATE OF ANALYSIS

**TO :**
**PRODUCT NAME : ADDZYME RD 165G**
**BATCH NO. : 111478-T**
**DATE OF MFD. : NOVEMBER, 2014**
**DATE OF EXPIRY : ***

### PROTOCOL OF ANALYSIS

| TEST | RESULT | LIMITS |
|---|---|---|
| **Description** | Cream coloured Granules | Off white to cream colored granules. |
| **Loss On Drying** | 5.37 % | Not More Than 7.0 % |
| **Microbial Limit –**<br>**E.coli**<br>**Salmonella** | <br>Absent in 1 g<br>Absent in 10 g | <br>Absent in 1 g<br>Absent in 10 g |
| **Enzyme Lipase Activity** | 268.55 BIU/g | Between 212 - 287 BIU/g |

**Remarks**: Sample **COMPLIES** as per Specifications
* THE PRODUCT WILL NOT LOSE MORE THAN 10 % ACTIVITY IN 12 MONTHS FROM THE DATE OF MFG. IF STORED IN 5°C.

(b) (6)

**QA-CHEMIST**
**Date: November 12, 2014**

(b) (6)

**MANAGER-QUALITY ASSURANCE**

## Appendix 3 - List of Reagents

*Exemplary list provided below*

| *Sr. No.* | *Reagent Name* | *Source* | *Catalogue No.* |
|---|---|---|---|
| *1.* | Palm Stearin | Kamani Oil Industries Pvt Ltd | - |
| *2.* | Refined Coconut oil-Parachute | Marico Ltd | - |
| *3.* | n-Hexane Emplura | Merck Specialities Pvt Ltd | *1.04368.2521* |
| *4.* | Potassium sulphate crystals Extra Pure | SD Fine Chemicals | *39658* |
| *5.* | Sodium Sulpate Dry purified | Merck Specialities Pvt Ltd | *61752105001730* |
| *6.* | Copper sulphate Pentahydrate Emplura | Merck Specialities Pvt Ltd | *1.93616.0521* |
| *7.* | Sulphuric acid 98% | Merck Specialities Pvt Ltd | *1.93400.2521* |
| *8.* | Methyl red 0.01% indicator solution | Merck Specialities Pvt Ltd | *61841301251730* |
| *9.* | Sodium hydroxide pellets (low Chloride) for analysis | Merck Specialities Pvt Ltd | *1.931020521* |

## Appendix 4 - List of Instruments

*Exemplary list provided below*

| *Sr. No.* | *Name of Instrument* | *Source* | *Instrument No.* |
|---|---|---|---|
| *1.* | Orbital Shaker cum incubator | Trishul Equipment | TRC-123 |
| *2.* | BioKjel-Biodist-AES (Semi-Automated Kjeldahl Equipment) | Techno Reach | TRC-127 |
| *3.* | Rotavap (Rotary evaporator | Superfit Continental Pvt. Ltd. | TRC-122 |
| *4.* | Balance-FX400 | Afcoset, Licence AND | TRC-108 |

## Appendix 5 - Procedure for Immobilization

Enzyme: Rhizopus Lipase produced from genetically modified Aspergillus niger agg. (strain FL100SC)
Carrier: Suitable carrier
Procedure:
The powder product is dissolved in water and the pH is adjusted. This liquid is added to carrier which has been pre-washed with distilled water and drained. The resultant enzyme-carrier slurry is stirred 27-30°C. Thereafter, the supernatant is decanted, and the wet beads are washed with distilled water and dried under vacuum.

## Appendix 6 - Procedure for Reaction

A model interesterification reaction was carried out with the enzyme.

Interesterification was carried out using 3.75g Palm Stearin, 11.25g Coconut oil and 45 ml n-Hexane. The mixture was equilibrated at 55°C to enable complete dissolution of the substrates. Suitable sample was withdrawn. The reaction was initiated by 1.5g of the immobilized enzyme and continued at 55°C in shaker cum incubator at 200 rpm for 4h. The enzyme was filtered off after the reaction. The samples, before the addition of enzyme and 4hrs post the reaction were subjected to solvent evaporation in a rotary evaporator. Thus obtained fat/oil samples were analysed for nitrogen/ protein content. All analyses were carried out in duplicate and averaged.

The nitrogen contents of the substrate and the reaction mass after 4 hours of reaction were analysed by using semi-automated Kjeldahl equipment for digestion and distillation, followed by manual titration. The nitrogen content was converted to protein content using 6.25 as conversion factor.

**Appendix 7 - Procedure for Nitrogen/protein estimation, if any.**

| Sr. No | Test | Method |
|---|---|---|
| 01 | Nitrogen content | • 0.3 g of sample are weighed and transferred to the digestion flask.<br>• 6 g of digestion mixture (100 g of sodium sulphate and 4 g of copper sulphate AR grade) and 20 ml of concentrate $H_2SO_4$ are added.<br>• This mixture is digested by heating about 60 – 90 minutes (until blue or slight greenish blue colour is observed) and cooled.<br>• 100 ml of distilled water and 6 – 8 glass beads are added and mixed carefully and thoroughly. The mixture is allowed to cool down to room temperature.<br>• 30% solution of NaOH is added to the digestion flask until a dark colour is observed.<br>• This flask is connected to the condenser with a water-cooling system.<br>• The digested mixture is heated and the condensate is collected in 40 ml of a 0.1 N HCl solution. The condensate outlet is carefully dipped into the HCl solution.<br>• TEST: About 100 ml of condensate are collected in a 0.1 N HCl solution and allowed to cool at room temperature. 5 to 6 drops of methyl-red are added as an indicator. This solution is titrated with 0.1 N NaOH solutions until colour changes from red to yellow. Volume is recorded as A (ml).<br>• BLANK: 40 ml 0.1 N HCl are measured. 5 to 6 drop of methyl-red are used as an indicator. This solution is titrated with 0.1 N NaOH solutions until colour changes from red to yellow. Volume is recorded as B (ml). |
| 02 | Calculation | $\text{Nitrogen content (\%w/w)} = \frac{(B - A) \times 0.14}{\text{Weight of sample}}$ |
| 03 | Protein Content | Protein Content (%) = % of nitrogen content X 6.38 |

**Gary Thundercliffe**
Innovation & Market
Development Director

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Bala Cynwyd, Pa 19004 USA
Telephone +1 610 668 9090
Fax + 1 610 668 8139

2nd April 2014

# Resolution ResAP(2004)3 Status for Purolite® Products

**Adsorbent & Enzyme Carrier Resins**

Products

| | | |
|---|---|---|
| Chromalite® PCG 900 | Purolite® AP1090 | Purolite® ECR 8310F |
| Macronet™ MN200 | Purolite® ECR 1090F | Purolite® ECR 8310M |
| Purosorb™ PAD 300 | Purolite® ECR 1090M | Purolite® ECR 8319F |
| Purosorb™ PAD 350 | Purolite® ECR 1091F | Purolite® ECR 8319M |
| Purosorb™ PAD 400 | Purolite® ECR 1091M | Purolite® ECR 8405F |
| Purosorb™ PAD 500 | Purolite® ECR 1504F | Purolite® ECR 8405M |
| Purosorb™ PAD 550 | Purolite® ECR 1504M | Purolite® ECR 8417F |
| Purosorb™ PAD 600 | Purolite® ECR 1508F | Purolite® ECR 8417M |
| Purosorb™ PAD 610 | Purolite® ECR 1508M | Purolite® ECR 8804F |
| Purosorb™ PAD 700 | Purolite® ECR 1604F | Purolite® ECR 8804M |
| Purosorb™ PAD 900 | Purolite® ECR 1604M | Purolite® ECR 8806F |
| Purosorb™ PAD 910 | Purolite® ECR 1640F | Purolite® ECR 8806M |
| Purosorb™ PAD 950 | Purolite® ECR 1640M | Purosep™ 200 |
| Purosorb™ PAD 950C | Purolite® ECR 8214F | |
| Purolite® ECR1030 | Purolite® ECR 8214M | |

These products comply with the regulations of the **COUNCIL OF EUROPE COMMITTEE OF MINISTERS' - Resolution ResAP(2004)3 on ion exchange and adsorbent resins used in the processing of foodstuffs**

The use of these products in food applications is strictly limited to the applications, restrictions, and operating requirements that are described in **Resolution ResAP(2004)3 on ion exchange and adsorbent resins used in the processing of foodstuffs.**

This document can be reviewed for the appropriate details at this Resolution ResAP(2004)3 website.

Pre-use treatment recommendations for Purolite ion exchange resins are available on our web site at:
Pre-use Conditioning of Ion Exchange Resins

Sincerely,

(b) (6)

Gary Thundercliffe
Innovation & Market Development Director

# Lifetech™ ECR8806M

Octadecyl methacrylate - Enzyme Carrier

PRODUCT DATA SHEET

**Lifetech ECR8806M** Octadecyl methacrylate is one of a range of enzyme carriers supplied under the **Purolite Lifetech ECR series**. The range includes different products each of standard particle size (M Grade) and small particle size (F Grade) which offer variations in type of immobilization, functional group, hydrophobicity and porosity. This product is designed for immobilization through adsorption of enzymes.

The adsorption method for the immobilization of an enzyme is based on the physical adsorption of enzyme protein on the surface of water-insoluble carriers. Hence, the method causes little or no conformational change of the enzyme or destruction of its active center. This method is particularly suitable for applications in organic solvents or hydrophobic media, as oils. Octadecyl-activated resins allow reversible but very strong adsorption of enzymes on these highly hydrophobic supports: enzymes may be desorbed after inactivation and the support may be reused. Adsorption on octadecyl-activated resins occurs via interfacial activation of the lipase on the hydrophobic supports at very low ionic buffer strength.

**Lifetech ECR8806M** Octadecyl methacrylate is an octadecyl (C18) activated resin with high porosity. This carrier is stable during storage and can be easily handled before and after immobilization procedures. **Lifetech ECR8806M** Octadecyl methacrylate is designed to strongly interact with enzymes and is optimal for lipases and transaminases.

**Lifetech ECR8806M** Octadecyl methacrylate is produced via a very intense crosslinking in the presence of a porogenic agent that allows the calibration of porosity.

**Lifetech ECR8806M** Octadecyl methacrylate is mechanically very stable and the final immobilized biocatalysts can be used in both stirred tank or bed reactor.

Performance of **Lifetech ECR8806M** Octadecyl methacrylate in the immobilization of enzymes are excellent compared to other commercial products and the immobilized enzymes can be used in organic solvent.

**Lifetech ECR8806M** is compliant with Resolution ResAP 2004 (3) on ion exchange and adsorbent resins used in the processing of foodstuffs.

### Typical Physical and Chemical Characteristics

| | |
|---|---|
| Application | Octadecyl methacrylate - Immobilization through adsorption of enzymes |
| Polymer Structure | Methacrylic |
| Appearance | Spherical Beads |
| Functional Group | Octadecyl |
| Ionic Form as Shipped | none |
| Particle Size Range | 300 - 710 µm |
| Surface Area | > 80 $m^2/g$ |

Purolite®

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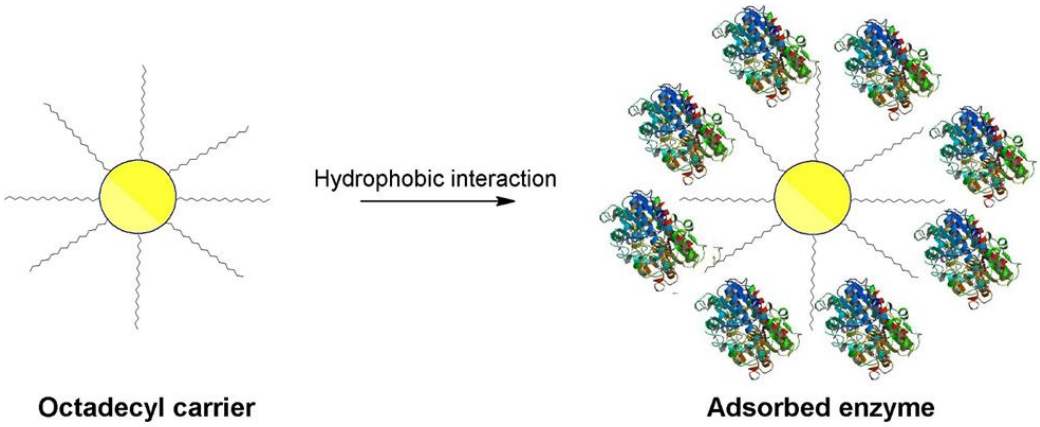
F +86 571 876 31385
asiapacific@purolite.com

# Lifetech™ ECR8806M

Octadecyl methacrylate - Enzyme Carrier

PRODUCT DATA SHEET

| | |
|---|---|
| d50, Meso and Macropores Å | 500 - 700 |
| pH limits, Stability | 2 - 10 |
| Average Breaking Weight | For prolonged stability store dry between 2°C - 20°C |

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08-SEP-2016
IIT STUDY NUMBER 17782

# 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 08-09-2016)

## IIT STUDY NUMBER 17782

## BACTERIAL REVERSE MUTATION TEST

## OF RHIZOPUS LIPASE

**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.

### CONFIDENTIAL

## CONTENTS

**CONTENTS (Contd.)**

# STATEMENT OF GLP COMPLIANCE

| | |
|---|---|
| Title of the Study | : Bacterial Reverse Mutation Test of Rhizopus Lipase |
| Study Number | : 17782 |
| Study Plan Number | : SPL/002/052 |
| Name of the Test item | : Rhizopus Lipase |

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. | (b) (6) | 08.09.2016 |
|---|---|---|
| Test Facility Management | Signature | Date |

## STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE

| | |
|---|---|
| Title of the Study | : Bacterial Reverse Mutation Test of Rhizopus Lipase |
| Study Number | : 17782 |
| Study Plan Number | : SPL/002/052 |
| Name of the Test item | : Rhizopus Lipase |

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.

This report is amended for addition of Study Schedule as per the study plan amendment number 1.

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. | (b) (6) | 08/09/2016 |
|---|---|---|
| Study Director | Signature | Date |

# STATEMENT OF QUALITY ASSURANCE UNIT

| | |
|---|---|
| Title of the Study | : Bacterial Reverse Mutation Test of Rhizopus Lipase |
| Study Number | : 17782 |
| Study Plan Number | : SPL/002/052 |
| Name of the Test item | : Rhizopus Lipase |

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 |
|---|---|---|---|
| 12/02/2014 | Study Plan Review | 12/02/2014 | 12/02/2014 |
| 15/04/2014 | Pre Study Verification | 15/04/2014 | 15/04/2014 |
| 16/04/2014 | Dose Range Finding Study | 16/04/2014 | 16/04/2014 |
| 19/04/2014 | Performance of Salmonella Reverse Mutation Assay (Expt.I) | 19/04/2014 | 19/04/2014 |
| 24/04/2014 | Revertant Bacterial Colony Count (Expt.II) | 24/04/2014 | 24/04/2014 |
| 30/04/2014 | Raw Data Audit | 30/04/2014 | 30/04/2014 |
| 12/06/2014 | Draft Report Audit | 12/06/2014 | 12/06/2014 |
| 30/06/2014 | Amendment 1 | 30/06/2014 | 30/06/2014 |
| 01/07/2014 | Final Report Audit | 01/07/2014 | 01/07/2014 |
| 08/09/2016 | Amended Final Report Review | 08/09/2016 | 08/09/2016 |

Mrs. C.S.Bhide M.Sc. — (b) (6) — 8.9.2016

Quality Assurance Unit — Signature — Date

## PERSONNEL INVOLVED IN THE STUDY

| | |
|---|---|
| Study Director | : Dr. R.P.Dighe Ph.D. |
| Study Scientists | : Mr. A.B.Sarvadnya M.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.<br>Mrs. C.S.Bhide M.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 |
| 2AA | - | 2-Aminoanthracene |
| 2AF | - | 2-Aminofluorene |
| DRF | - | Dose Range Finding |
| DMF | - | Dimethyl Formamide |
| DMSO | - | Dimethyl sulphoxide |
| Expt. | | Experiment |
| GLP | - | Good Laboratory Practice |
| IIT | - | Indian Institute of Toxicology |
| kg | - | Kilogram |
| mg | - | Milligram |
| Ml | - | Milliliter |
| MMS | - | Methyl methanesulphonate |
| No. | - | Number |
| Nos. | - | Numbers |
| NQNO | - | 4-Nitroquinolene-N-Oxide |
| OECD | - | Organization for Economic Co-operation and Development |
| S9 | - | Supernatant at 9000 g |
| SOP | - | Standard Operating Procedure |

## SUMMARY AND CONCLUSION

In order to determine the potential of the test item **Rhizopus Lipase** 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/plate to 5000 µg/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/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/plate, 185.18 µg/plate, 555.55 µg/plate, 1666.66 µg/plate and 5000 µg/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 sodium phenobarbital and β-naphthoflavone induced rat liver. Untreated control, vehicle control (negative control) and appropriate positive controls (Methyl methane sulphonate, Sodium azide, 4-Nitroquinolene-N-Oxide for without metabolic activation and 2-Aminofluorene, 2 Aminoanthracene and Danthron 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 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 3.38 to 36.97 fold under identical conditions.

**Conclusion**

Based on these results it is concluded that **Rhizopus Lipase** supplied by **Advanced Enzyme Technologies Ltd., Thane,** when tested at and up to 5000 µg/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.

(b) (6)

**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 | : 12-02-2014 |
| Experimental Starting Date | : 16-04-2014 |
| **Range Finding Study -** | |
| Experimental Starting Date | : 16-04-2014 |
| Experiment Completion Date | : 18-04-2014 |
| **Definitive Study -** | |
| **Experiment I** | |
| Experimental Starting Date | : 19-04-2014 |
| Experiment Completion Date | : 21-04-2014 |
| **Experiment II** | |
| Experimental Starting Date | : 22-04-2014 |
| Experiment Completion Date | : 24-04-2014 |
| Study Completion Date | : 01-07-2014 |
| Date of Reporting | : 01-07-2014 |
| Final Report Amendment Date | : 08-09-2016 |

## Archives

All original raw data, the signed study plan and a copy of final report is 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 were 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

This study was designed to evaluate **Rhizopus Lipase** and/or its metabolites for their ability to induce reverse mutation at the histidine locus in the genome of 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 | : **Rhizopus Lipase** |
| Batch Number | : **011423** |
| Laboratory Test Item Code | : **TAS/002/015** |
| Manufacturing Date | : **January, 2014** |
| Expiry Date | : **December, 2015** |
| Consistency | : **Solid powder** |
| Activity (Clinical Indication) | : **Food Enzyme** |
| Safety Precautions | : Safety precautions included use of protective clothing, gloves, masks and eye protection (glasses). |
| Stability Data | : Information on file. |
| Storage Condition | : Ambient temperature |
| Dose Preparation | : Test item was dissolved in distilled water and diluted to required concentrations of the test item prior to exposure of cell cultures. |
| 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

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

1) In order to meet the regulatory requirement for testing,
2) Availability of comprehensive background data at the test facility,
3) Widely used as a preferred strain of choice in genetic toxicological studies,
4) These strains are widely used throughout the industry in the genotoxicity studies.

**Source:**

Strains were procured from Moltox Inc., USA.

**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 battery 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:

Nutrient broth (Hi Media) to grow overnight cultures. Minimal Glucose Agar for mutagenicity assay. Top agar with trace of histidine and biotin for selection of histidine revertants.
Minimal Glucose Agar plates are incubated at 37°C for 24 hours after preparation to check the sterility of plates. The plates showing colonies of contaminants on agar are discarded.
Top Agar with histidine biotin solution.

Overnight Bacterial Cultures:

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. Excessive aeration of overnight cultures was avoided. Overnight shaking of cultures in flasks did not exceed 120 rpm.

The cultures used in the experiment contained a high titer of viable bacteria. The titer was demonstrated either from historical control data or in each assay through the determination of viable cell count.

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.

### Control Item:

Vehicle control (Negative control):

Distilled water was plated for all strains with and without microsomal enzymes.

Positive control:

Positive controls with and without microsomal enzymes were plated as follows:

Without metabolic activation:

1. Methyl methanesulphonate (MMS) 1 µl/plate, for TA100 and TA102 tester strains.
2. 4-Nitroquinolene-N-Oxide (NQNO) 0.5 µg/plate for TA 97a and TA98
3. Sodium azide 0.5 µg/plate for TA 1535

With metabolic activation (with S-9):

1. 2-Aminoanthracene (2AA) 0.5 µg/plate for TA 1535
2. 2-Aminofluorene (2AF) 10 µg/plate, for TA 97a, TA 98 TA 100.
3. Danthron 30 µg/plate forTA102.

**Untreated control (Spontaneous Revertant Data):**

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

**Preparation of S-9 Mix:**

The S9 fraction prepared on date 10-12-2013, batch number 11 was used in this study.

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

Following co-factors were added to the S-9 fraction:

| 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:**

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 or less 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 was inverted and incubated for 48 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 haemocytometer 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 in the presence and absence of microsomal enzymes.

2) Tester strain TA 100 was used for cytotoxicity study because the growth inhibitory effect of TA 100 is generally representative of that observed on other tester strain. Similarly TA 100 is known to show 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/plate were assessed. Distilled water was used as Vehicle control (Negative control).

**Main Test:**

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

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

**Main Test (Experiment I and Experiment II):**

The S-9 mix (0.5 ml) or phospate buffered saline (PBS) pH 7.4, 0.1 ml of bacterial culture and 0.1 ml or less 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 was inverted and incubated for 48 hour at 37 °C. All plating was done in triplicate. The experiment was repeated once for consistent results.

## 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 at least twice the concurrent Vehicle controls with or without some evidence of positive dose relationship, in two separate experiments, with any bacterial strain either in the presence or absence of S-9 mix, it 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 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. Simple linear regression analysis was performed for each strain separately to determine any dose dependent increase in the mean revertant colonies.

The revertant colonies for untreated control, vehicle control and positive control groups fall within the in-house range. 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 be 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 was conducted within a month.
- The tester strain cultures exhibited a characteristic number of spontaneous revertants per plate. The number of spontaneous revertants was expected to fall within the spontaneous mutation rate at this laboratory, although they may occasionally fall outside of this range. In such a case the experiment 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 material 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/plate.

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

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

The mean number of histidine revertants in the vehicle control was 109.00 and 109.33 without and with metabolic activation, respectively. In the highest concentration of 5000 µg/plate the mean number was 96.67, 107.33 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/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 : 11.00, 12.00; TA97a : 105.00, 106.33; TA 98 : 28.33, 30.00; TA 100 : 106.67, 109.00 and TA 102 : 291.33, 306.00 without and with metabolic activation. In the highest concentration of 5000 µg/plate the mean number was 8.00, 9.67, 93.33, 100.00, 26.33, 28.00, 105.33, 107.00 and 284.67, 275.33 without and with metabolic activation 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 3.62 to 36.97 and 3.38 to 35.60 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 : 11.33, 12.33; TA97a : 104.67, 106.33; TA 98 : 26.67, 29.00; TA 100 : 107.00, 109.00 and TA 102 : 285.33, 301.33, without and with metabolic activation. In the highest concentration of 5000 µg/plate the mean number was 8.33, 10.33, 94.67, 100.00, 26.00, 27.33, 104.33, 107.00 and 284.00, 278.00 without and with metabolic activation 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. The parallel positive controls induced 3.62 to 35.95 and 3.96 to 36.69 fold revertants without and with metabolic activation in comparison with respective vehicle controls.