



**Advanced Enzyme Technologies Ltd.**

CIN: L24200MH1989PLC051018

Sun Magnetica, 'A' wing, 5th Floor, LIC Service Road, Louiswadi, Thane (W)-400 604, India

Tel: +91-22-4170 3200, Fax: +91-22-2583 5159

Email: [info@advancedenzymes.com](mailto:info@advancedenzymes.com), [www.advancedenzymes.com](http://www.advancedenzymes.com)

GRN # 783

Date: 26<sup>th</sup> April 2018  
Center for Food Safety and Applied Nutrition  
Food and Drug Administration  
5001 Campus Drive  
College Park, MD 20740

**Subject:** GRAS notification for "Lipase from genetically modified *Aspergillus niger* agg. (strain FL100SC) for modification/esterification of lipids"

Dear Sir/Madam,

We, Advanced Enzymes Technologies Ltd, are submitting a virus free CD containing GRAS notification along with form 3667 for Lipase from genetically modified *Aspergillus niger* agg. (strain FL100SC) for modification/esterification of lipids (more specifically, but not limited to human milk substitutes and cocoa butter substitutes) for review by the FDA. The CD also contains answers to the questions raised by USFDA in the letter dated 10th October, 2017. We believe that this determination and notification are in compliance with 21 C.F.R. part 170, subpart E on "Generally Recognised as Safe (GRAS) notice".

Please feel free to contact us if additional information or clarification is needed as you proceed with the review. We would appreciate your kind attention to this matter.

Sincerely yours,

(b) (6)

Deepti Sood  
Manager-IP & Regulatory Affairs  
Email ID: [deepti@advancedenzymes.com](mailto:deepti@advancedenzymes.com)

**FDA USE ONLY**

DEPARTMENT OF HEALTH AND HUMAN SERVICES  
Food and Drug Administration

**GENERALLY RECOGNIZED AS SAFE  
(GRAS) NOTICE** (Subpart E of Part 170)

GRN NUMBER	DATE OF RECEIPT
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway (see Instructions); OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety (HFS-200), Center for Food Safety and Applied Nutrition, Food and Drug Administration, 5001 Campus Drive, College Park, MD 20740-3835.

**SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION**

1. Type of Submission (Check one)  
 New       Amendment to GRN No. 708       Supplement to GRN No. \_\_\_\_\_

2.  All electronic files included in this submission have been checked and found to be virus free. (Check box to verify)

3. Most recent presubmission meeting (if any) with FDA on the subject substance (yyyy/mm/dd): \_\_\_\_\_

4. For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? (Check one)  
 Yes If yes, enter the date of communication (yyyy/mm/dd): \_\_\_\_\_  
 No communication (yyyy/mm/dd): \_\_\_\_\_

**SECTION B – INFORMATION ABOUT THE NOTIFIER**

<b>1a. Notifier</b>	Name of Contact Person Mr. Piyush Rathi	Position or Title Chief Business Officer	
	Organization (if applicable) Advanced Enzyme Technologies Ltd		
	Mailing Address (number and street) 5th Floor, Sun Magnetica, LIC Service Road, Louiswadi		
City Thane west	State or Province Maharashtra	Zip Code/Postal Code 400604	Country India
Telephone Number +91 2241703200	Fax Number	E-Mail Address piyush@advancedenzymes.com	
<b>1b. Agent or Attorney (if applicable)</b>	Name of Contact Person	Position or Title	
	Organization (if applicable)		
	Mailing Address (number and street)		
City	State or Province	Zip Code/Postal Code	Country
Telephone Number	Fax Number	E-Mail Address	



**SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE**

*(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)*

- PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).
- PART 3 of a GRAS notice: Dietary exposure (170.235).
- PART 4 of a GRAS notice: Self-limiting levels of use (170.240).
- PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).
- PART 6 of a GRAS notice: Narrative (170.250).
- PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

**Other Information**

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

Yes     No

Did you include this other information in the list of attachments?

Yes     No

**SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS**

1. The undersigned is informing FDA that Mr. Piyush Rathi  
(name of notifier)  
 has concluded that the intended use(s) of Triacylglycerol lipase from Rhizopus oryzae produced by genetically modified Aspergillus niger  
(name of notified substance)  
 described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. Mr. Piyush Rathi  
(name of notifier) agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.

Advanced Enzyme Technologies Ltd, 5th Floor, Sun Magnetica, LIC Service Road, Louiswadi, Thane-400604, India  
(address of notifier or other location)

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance. The notifying party certifies that the information provided herein is accurate and complete to the best of his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official, Agent, or Attorney <b>(b) (6)</b>	Printed Name and Title Mr. Piyush Rathi, Chief Business Officer	Date (mm/dd/yyyy) 04/10/2018
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**PART VIII – LIST OF ATTACHMENTS** *(continued)*

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Identification of production strain (Annex H1)	Folder containing Annexes
	Identification of parental organism (Annex H2)	Folder containing Annexes
	Allergenicity Assessment - Rhizopus oryzae lipase produced by Aspergillus niger agg (strain FL100SC) (Annex I)	Folder containing Annexes
	Report on assessment of the leakage of active enzyme and / or immobilisation support materials/resins into food (Annex J)	Folder containing Annexes
	Combined file of toxicity studies: Bacterial reverse mutation test of Rhizopus oryzae lipase (Annex K)	Folder containing Annexes
	In vitro mammalian chromosome aberration test of Rhizopus oryzae lipase in human lymphocytes (Annex K)	Folder containing Annexes
	Repeated dose 90-day oral toxicity study with Rhizopus oryzae lipase by daily gavage in the rat followed by a 4 week recovery period (Annex K)	Folder containing Annexes
	Toxicity reports of Pectin lyase, Pectin (methyl) esterase, polygalacturonase and pectinase (Annex L)	Folder containing Annexes
	Expert Panel Report (Annex M)	Folder containing Annexes

Add Continuation Page

**OMB Statement:** Public reporting burden for this collection of information is estimated to average 170 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services, Food and Drug Administration, Office of Chief Information Officer, [PRASStaff@fda.hhs.gov](mailto:PRASStaff@fda.hhs.gov). (Please do NOT return the form to this address.). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.

# GRAS NOTIFICATION

**Triacylglycerol lipase from *Rhizopus oryzae* produced by genetically modified *Aspergillus niger* agg. (strain FL100SC) for modification/esterification of lipids**



Advanced Enzyme Technologies Ltd.  
5th Floor, 'A'-wing, Sun Magnetica,  
L.I.C. Service Road, Louiswadi,  
Thane – 400 064, INDIA

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# **§ 170.225 PART 1, GRAS NOTICE: SIGNED STATEMENTS AND CERTIFICATION**

## **1.1 GRAS NOTICE SUBMISSION**

We submit this GRAS notice in accordance with 21 C.F.R. part 170, subpart E.

## **1.2 NAME AND ADDRESS OF NOTIFIER**

### **APPLICANT**

Name: Advanced Enzyme Technologies Ltd.  
Address: 5th Floor, Sun Magnetica LIC Service Road, Louiswadi  
Postal code and City: Thane West, India 400604  
Country: India  
Tel. no: +91 22 41703200  
Fax no: +91 22 25835159  
E-mail: [info@advancedenzymes.com](mailto:info@advancedenzymes.com)

### **MANUFACTURER**

Name Company: Advanced Enzyme Technologies Ltd  
Address: 5th Floor, Sun Magnetica LIC Service Road, Louiswadi  
Postal code and City: Thane West, India 400604  
Country: India  
Tel. no: +91 22 41703200  
Fax no: +91 22 25835159

### **PERSON RESPONSIBLE FOR THE DOSSIER**

Name Person: Piyush Rathi  
Chief Business Officer  
Advanced Enzyme Technologies Ltd.  
Address: 5th Floor, Sun Magnetica LIC Service Road, Louiswadi  
Postal code and City: Thane West, India 400604  
Country: India  
Tel. no: +91 22 41703200  
E-mail: [piyush@advancedenzymes.com](mailto:piyush@advancedenzymes.com)

## **1.3 NAME OF NOTIFIED SUBSTANCE**

The name of the substance that is the subject of this GRAS determination is Triacylglycerol lipase – EC 3.1.1.3 [*Rhizopus* lipase produced by genetically modified by *Aspergillus niger* agg. (Strain FL100SC)]

*For the ease of reference, the Rhizopus lipase produced by genetically modified Aspergillus niger agg. (strain FL100SC) has been presented by the common/ shorter names: Lipase, ROL, Rhizopus oryzae lipase or Rhizopus delemar lipase or Rhizopus lipase, lipase from Aspergillus niger agg. (strain FL100SC), Rhizopus lipase from genetically modified A.niger, throughout the dossier and related annexes.*



#### 1.4 APPLICABLE CONDITIONS OF USE

Lipase is to be used in modification/esterification of fats & oils. The enzymatic conversion of lipids with the help of the lipase can be used in the processing of all food raw materials which naturally contain lipids.

For an enzyme to perform a technological function in the final food, certain conditions have to be met, such as the enzyme must be in its native, non-denatured form, a substrate must be present and conditions such as pH, temperature and water content must be favourable for the particular enzyme.

Suitable conditions for eliciting the lipase activity are:

pH range: 5.0-9.0

Temperature range: 25°C – 45°C.

Temperature range for immobilized enzymes: 20°C - 60°C

##### 1.4.1 SUBSTANCE USED IN

*Rhizopus* lipase from *Aspergillus niger* agg. (strain FL100SC) performs its technological function during food processing. It is mainly intended to be used in modification / esterification of lipids.

More specifically, the products intended to be synthesized using *Rhizopus* lipase are included, but not limited to the below mentioned speciality lipid products:

1. Human milk fat substitutes
2. Cocoa butter substitutes/equivalents

##### 1.4.2 LEVELS OF USE

In principle, the enzymatic conversion of glycerides present in fats & oil with the help of lipase can be used in the processing of all food raw materials which naturally contain these substrates.

The lipase covered in this dossier is typically used in modification / esterification of lipids, more specifically for the preparation of human milk substitutes and cocoa butter equivalents.

*Rhizopus* lipase is immobilized on an inert carrier. The weight percent of *Rhizopus* lipase immobilized on the carrier is between 5-10%. Typically, 0.3-1kg of immobilized *Rhizopus* lipase granules is used per ton of oil/fat.

The *Quantum Satis* principle is used by food manufacturers in relation to food enzyme preparations, which means that food manufacturers will typically optimise the enzyme dosage based on a dose range recommended by the enzyme supplier.

##### 1.4.3 PURPOSES

The food enzyme object of this dossier, *inter alia*, improves physicochemical properties (e.g. melting point, consistency, firmness, etc.), aids in preparation of nutritionally important value added products, reduces long chain fatty acids, produces *trans* fat-free products, and replaces conventional chemical esterification with eco-friendly enzymatic alternative.(Sharma et al. 2001).



*Rhizopus* lipase from *Aspergillus niger* agg. (Strain FL100SC) performs its technological function during food processing, be it the preparation of human milk substitutes or the preparation of cocoa butter equivalents. It is mainly intended to be used as a processing aid in modification / esterification of lipids.

#### **1.4.4 CONSUMER POPULATIONS**

Lipase is ubiquitous in nature and is present in microorganisms, and most notably in plants and animals consumed by humans (Sharma et al, 2001; Aravindan et al., 2007). This implies that lipase is abundant in human diet and will be digested like any other protein in the human body. Hence the addition of *Rhizopus* lipase in any of proposed applications will have no significant effect on the human body.

Importantly, lipase performs its technological function during food processing and does not perform any technological function in the final food. The enzyme used in food processing is immobilized on the carrier and is unlikely to be present in the final food in the active form. The product post enzymatic reaction is subjected to distillation, filtration and deodorization. Owing to the foregoing reasons, even the traces of enzymes released in the final product will not contain any active enzyme residues.

Further, *Rhizopus* lipase enzyme and its production host *Aspergillus niger* spp. have a long history of use in food processing. *Rhizopus* lipase is approved by Health Canada, Brazil Food Authority, Légifrance, GB list of China and Food standards of Australia and New Zealand. Also, FDA, had no questions regarding the conclusion that *Rhizopus* lipase enzyme preparation is GRAS under the intended conditions of use. (GRN No. 000216).

In the early 1960s the FDA issued opinion letters recognizing, inter alia, that enzymes from *Aspergillus niger* can be ‘generally regarded as safe’ (GRAS) under the condition that non-pathogenic and non-toxicogenic strains and current good manufacturing practices be used in production (Schuster et al 2002). In the Food Standards Code-1.3.3-processing aids of FSANZ, lipase from *Aspergillus niger* is listed as safe for use in food.

Overall, the above points indicate that consumer population will not be affected by the presence of lipase in food preparation(s).

#### **1.5 STATUTORY BASIS FOR GRAS DETERMINATION**

Advanced Enzyme Technologies Ltd., hereby notifies FDA of the submission of a GRAS notice, meeting the specifications described herein, has been determined to be GRAS through scientific procedures in accordance with § 170.30(a) and (b).

#### **1.6 PREMARKET APPROVAL STATEMENT**

Advanced Enzyme Technologies Ltd. further asserts that the use of the notified substance is exempt from pre-market approval requirements of the Federal Food, Drug, and Cosmetic Act based on a conclusion that the notified substance is GRAS under the conditions of its intended use.

**1.7 AVAILABILITY OF INFORMATION**

Advanced Enzyme Technologies Ltd agrees to make the data and information that is the basis for the determination of GRAS status available to FDA if FDA asks to see them.

Upon FDA's request, Advanced Enzyme Technologies Ltd agrees to allow FDA to review and copy these data and information during customary business hours at the address mentioned above.

Upon FDA's request, Advanced Enzyme Technologies Ltd agrees to send these data and information to FDA either in electronic format or on paper.

**1.8 APPLICABILITY OF FOIA EXEMPTIONS**

None of the data and information in Part 2 through 7 of our GRAS notice are exempt from disclosure under the Freedom of Information Act, 5 U.S.C. § 552.

**1.9 GRAS NOTICE CERTIFICATION**

To the best of our knowledge, the GRAS notice is complete, representative, and balanced submission that includes information, known to us and pertinent to the evaluation of the safety and GRAS status of the use of the substance

**1.10 NAME/POSITION OF NOTIFIER**

Mr. Piyush Rathi.  
Chief Business Officer.  
Advanced Enzyme Technologies Ltd.  
5th Floor, Sun Magnetica LIC Service Road, Louiswadi  
Thane West, India 400604  
Tel. no: +91 22 41703200  
E-mail: [piyush@advancedenzymes.com](mailto:piyush@advancedenzymes.com)

(b) (6)

Signature:

Date: 04/10/2018

**2 § 170.225 PART 2, GRAS NOTICE: IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT**

**2.1 ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE**

**2.1.1 ENZYME IDENTITY**

Name of the enzyme protein:	Triacylglycerol lipase
Synonyms:	Triacylglycerol acylhydrolases; Lipase; Tributyrase; Triglyceride lipase; Glycerol ester hydrolase.
Abbreviations:	ROL
EC (IUBMB) number:	<a href="#">EC 3.1.1.3</a>
Reaction catalysed:	Hydrolysis of carboxylic ester bonds in glycerides and producing free fatty acids, diacylglycerols, monoacylglycerols and glycerols. In addition, lipase also catalyzes the esterification of fatty acids and alcohols or rearranges fatty acids in glycerides under micro-aqueous condition.

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

EC 3	Hydrolases
EC 3.1	Acting on ester bonds.
EC 3.1.1	Carboxylic ester hydrolases
EC 3.1.1.3	Triacylglycerol lipase

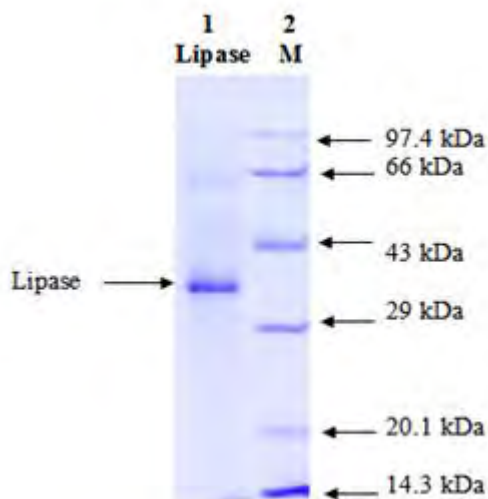
*For the ease of reference triacylglycerol lipase from Rhizopus oryzae produced by genetically modified Aspergillus niger agg strain (FL100SC) has been presented by the common/ shorter name: Lipase, Rhizopus oryzae lipase, or Rhizopus delemar lipase or Rhizopus lipase, lipase from Aspergillus niger agg. (strain FL100SC) throughout the dossier.*

### 2.1.2 MOLECULAR MASS, SUBUNIT STRUCTURE AND AMINO ACID SEQUENCE OF THE ENZYME PROTEIN

The molecular weight of lipase preparation from *Aspergillus niger* agg. (strain FL100SC), using SDS-PAGE, was determined to be 37.8 kDa. This is in close agreement with information obtained by searches conducted on freely available protein databases like [Brenda](#) and [UniProtKB](#).

The methods by which the electrophoretic data was obtained are given in [Annex B](#).

The protein profile of the *Rhizopus* lipase from *Aspergillus niger* agg. (strain FL100SC) is illustrated by the electrophoretic data given below:



Lane 1-Lipase = *Rhizopus* lipase; Lane 2-M: Molecular Weight Marker

Figure 2.1.2-1 Electrophoretic data of *Rhizopus* lipase preparation from *Aspergillus niger* agg. (strain FL100SC) (Batch No 0413134)

The lipase protein from *Aspergillus niger* agg. (strain FL100SC) comprises 392 amino acids with corresponding molecular mass of 37.8 kDa, determined using SDS-PAGE. Molecular weight determined using gel permeation chromatography provides suggestive evidence that lipase protein is a monomer.

The above information is in agreement with information available on protein databases such as [ExPASy](#), [Brenda](#) and [UniProtKB](#) (UNIPROT P61872), provided below is the sequence:

```
MVSFISISQGVSLCLLVSSMMLGSSAVPVSGKSGSSNTAVSASDNAALPPLISSRCAPPS
NKGSKSDLQAEPYNMQKNTEWYESHGGNLTSGKRDDNLVGGMTLDLPSDAPPISLS
SSTNSASDGGKVVAATTAQIQEFTKYAGIAATAYCRSVVPGNKWDCVQCQKWVPDG
KIITFTSLLSDTNGYVLRSDKQKTIYLVFRGTNSFRSAITDIVNFSDYKPVKGAKVH
AGFLSSYEQVVNDYFPVVQEQLTAHPTYKVIIVTGHSLGGAQALLAGMDLYQREPLRS
PKNLSIFTVGGPRVGNPTFAYYVESTGIPFQRTVHKRDIVPHVPPQSFGLHPGVESWI
KSGTSNVQICTSEIETKDCSNSIVPFTSILDHLSYFDINEGSCL
```



### 2.1.3 **ENZYME ACTIVITY**

Lipase (IUBMB 3.1.1.3) catalyzes hydrolysis of carboxylic ester bonds in triacylglycerols and producing free fatty acids, diacylglycerols, monoacylglycerols and glycerols. In addition, lipase catalyzes the esterification of fatty acids and alcohols or rearranges fatty acids in glycerides under micro-aqueous condition. (Houde et. al., 2004; Aravindan et al. 2007) Substrates mainly include glycerides (triglycerides, diglycerides, and monoglycerides), fatty acids, and glycerol present in fats and oils.

The method to analyze the activity of enzyme is based on internationally recognized Food Chemical Codex (FCC) assay (Refer [Annex A2](#)). The immobilized enzyme granules are assayed using company specific in house method.

To the best of our knowledge, the *Rhizopus oryzae* lipase from genetically modified *Aspergillus niger* agg. (strain FL100SC), described in this dossier does not possess any enzymatic side activities which might cause adverse effects.

### 2.1.3.1 Information on the pH and temperature optima of the food enzyme

The activity of the food enzyme *Rhizopus* lipase from *Aspergillus niger* agg. (strain FL100SC) was measured under various pH and temperature conditions.

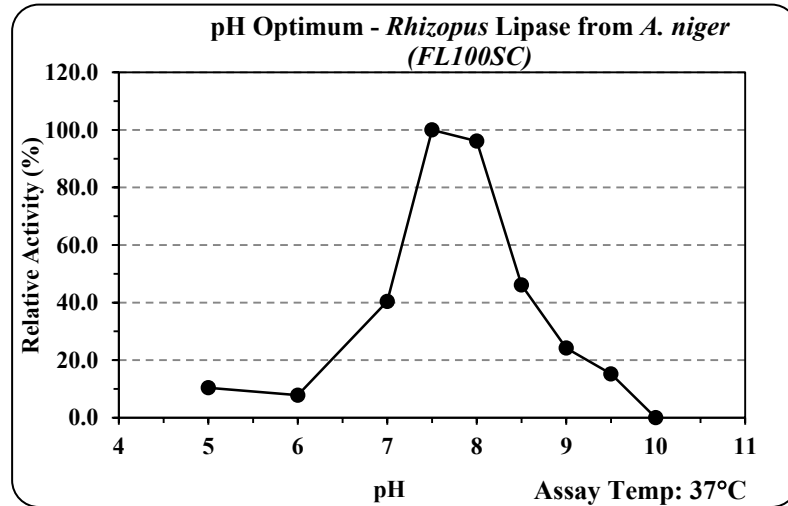


Fig: 2.1.3-1: pH Optimum of *Rhizopus* lipase from *Aspergillus niger* agg. (strain FL100SC)

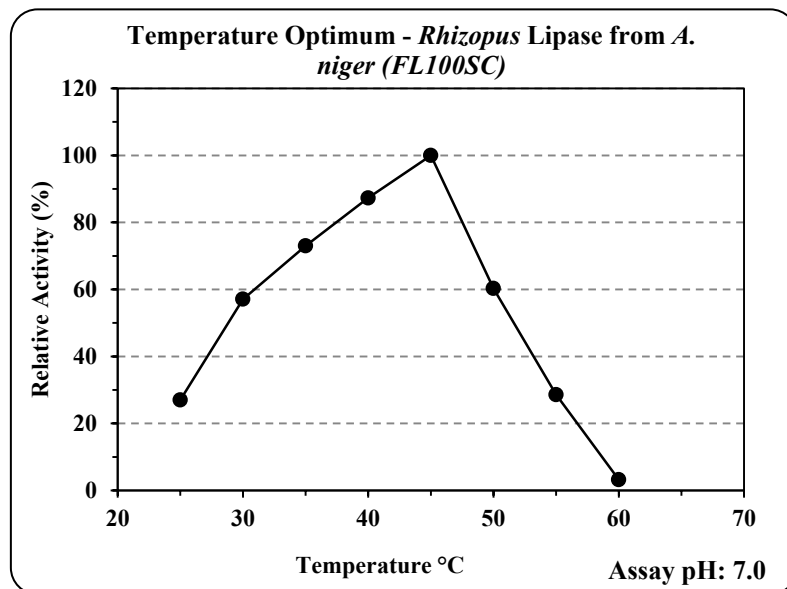


Fig: 2.1.3-2: Temperature Optimum of *Rhizopus* lipase from *Aspergillus niger* agg. (strain FL100SC)

As can be concluded from the above Figures 2.1.3-1 and 2.1.3-2, *Rhizopus oryzae* lipase produced by genetically modified *Aspergillus niger* agg. (strain FL100SC) exhibits activity, under test conditions, from pH 5.0 till pH 9.5 and from 25°C till 60°C in the presence of olive oil substrate. The optimum pH range lies between pH 7.5 and 8.0, with the maximum activity at pH 7.5. Likewise, the optimum temperature range lies between 40 and 45°C, with the maximum activity at 45°C. (Refer [Annex C](#) for details)

### 2.1.3.2 Information on the stability of the food enzyme

The stability of the food enzyme *Rhizopus* lipase from *Aspergillus niger* agg. (strain FL100SC) was measured under various pH and temperature conditions.

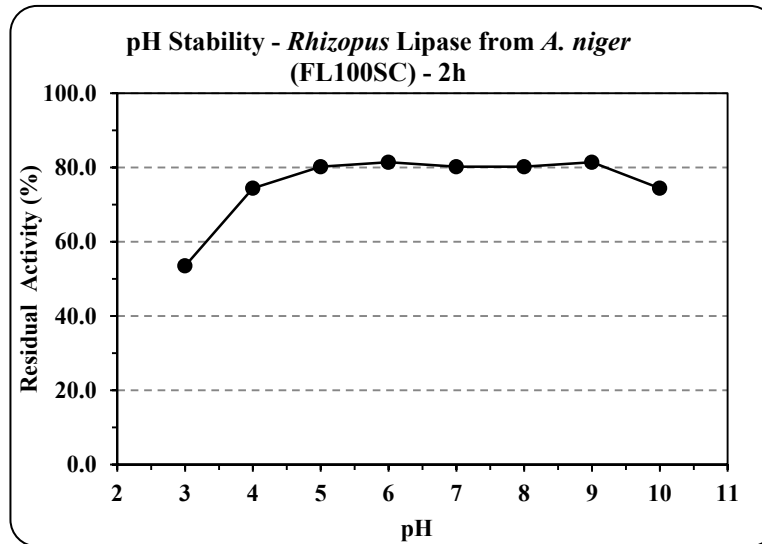


Fig: 2.1.3-3: pH stability of *Rhizopus* lipase from *Aspergillus niger* agg. (strain FL100SC)

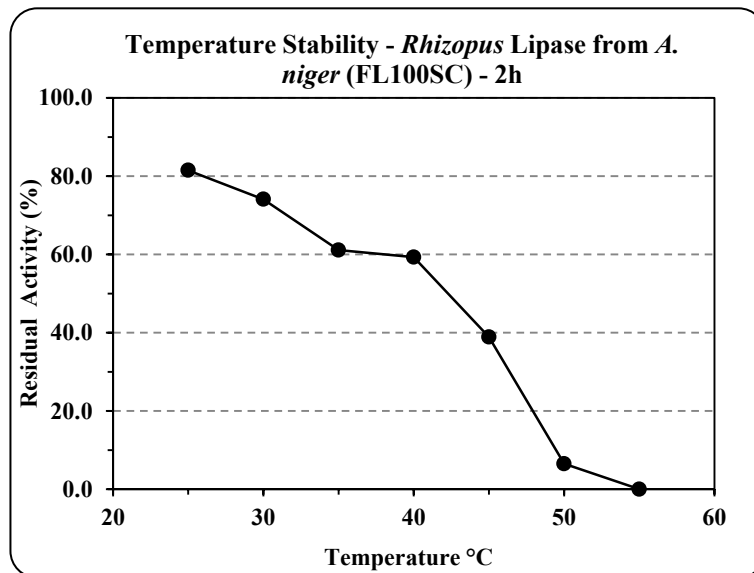


Fig: 2.1.3-4: Temperature Stability of *Rhizopus* lipase from *Aspergillus niger* agg. (strain FL100SC)

It is clear from the figure 2.1.3-3 that *Rhizopus oryzae* lipase from *Aspergillus niger* agg. (strain FL100SC) was stable between pH 5.0 to 9.0 for 2 hours.

Figure 2.1.3-4 shows that lipase enzyme was found to be stable upto 40°C for 2 hours. The enzyme activity decreases rapidly at temperatures higher than 45°C. (Refer [Annex C](#) for details)

### 2.1.3.3 Information on the temperature stability of the immobilized food enzyme in n-Hexane

The immobilized enzyme is intended for use in non-aqueous media. Hence, its stability was evaluated in n-Hexane.

Conditions of study: The immobilized beads were incubated in n-Hexane at mentioned temperature for 1hour. n-Hexane was then decanted and the beads were dried under vacuum. This was followed by the in-house activity assay. The Relative activity with respect to temperature is expressed in the figure below.

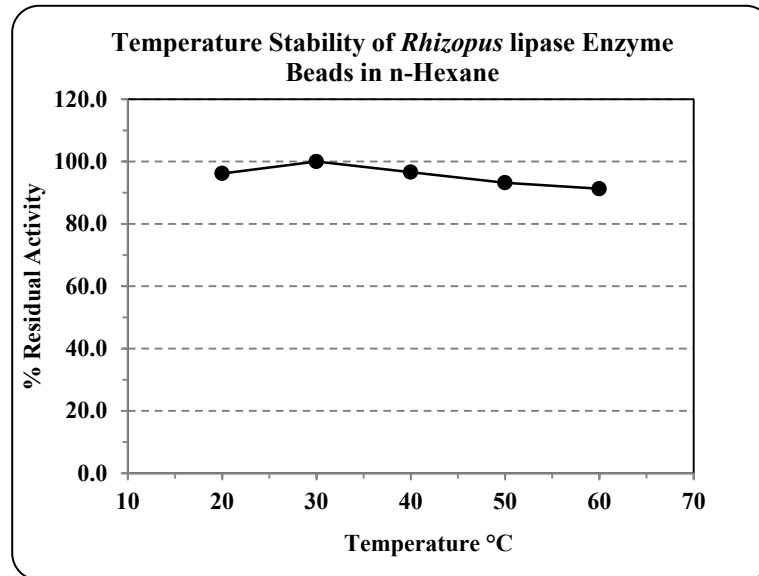


Fig: 2.1.3-5: Temperature stability of immobilized *Rhizopus* lipase from *Aspergillus niger* agg.(strain FL100SC)

It is evident from figure 2.1.3-5 that *Rhizopus* lipase enzyme beads are stable in n-hexane between 20-60 °C



## 2.2 PRODUCTION ORGANISM

### 2.2.1 SCIENTIFIC NAME, TAXONOMY AND OTHER NAMES

The microorganism that is used for the production of the lipase is genetically modified *Aspergillus niger* agg. (strain FL100SC), which is derived from the parental strain *Aspergillus niger* (strain ASNSC). The parental strain was obtained by commercial cooperation from a company in 1992.

#### Scientific name:

Genus: *Aspergillus*

Species: Section *Nigri* species\*\*

\*\* *Centraalbureau voor Schimmelcultures; NL (CBS) in their assessment concluded the parental strain ASNSC to be Aspergillus Section Nigri species, and that strain shared sequences with isolates of Aspergillus acidus, Kozak. (redesignated as Aspergillus luchuensis by Hong et al, 2013). Further, based on bioinformatic analysis of sequence data [partial fragment of  $\beta$ -tubulin (BT) gene and partial fragment of calmodulin (CMD) gene], the production strain FL100SC has been shown to share sequences with isolates of Aspergillus luchuensis (earlier known as Aspergillus acidus Kozak.), and shown not to produce any mycotoxins as tested by CBS. Importantly, Hong et al, 2013 reported that Aspergillus luchuensis does not produce any mycotoxins. This is in agreement with studies conducted on parental strain ASNSC and production strain FL100SC, by CBS, wherein both strains were found not to produce mycotoxins.*

*Interestingly, another leading Mycology centre - Center for Agriculture and Biosciences International; UK (CABI) – based on its findings has concluded that parental strain ASNSC be designated as Aspergillus niger agg..*

*In view of the above findings, the production strain was designated as Aspergillus niger agg. (strain FL100SC).*

#### Taxonomy:

Kingdom	:	Fungi
Phylum	:	Ascomycota
Sub-phylum	:	Pezizomycotina
Class	:	Eurotiomycetes
Sub-class	:	Eurotiomycetidae
Order	:	Eurotiales
Family	:	Trichocomaceae
Genus	:	<i>Aspergillus</i>
Species	:	Section <i>Nigri</i> species

*The working name of the source material (production strain) during studies conducted for the preparation of the dossier was Aspergillus niger or Aspergillus niger agg (strain FL100SC). Therefore the names Aspergillus niger or Aspergillus niger (strain FL100SC) appear in certain Annexes to the dossier. Likewise, the terms Aspergillus niger agg. (strain ASNSC) and Aspergillus niger (strain ASNSC) are used interchangeably while referring to the parental strain, in certain sections of the dossier.*

## 2.2.2 DONOR, RECIPIENT ORGANISM AND PRODUCTION STRAIN

### Donor

The *Rhizopus* lipase gene (*lip3-prepro*) used to genetically modify the recipient *Aspergillus niger* agg. (strain ASNpyrE#8) is a synthetic gene encoding the protein sequence of *Rhizopus oryzae* lipase. The Coding DNA Sequence of the *Rhizopus oryzae* *lip3-prepro* gene was codon optimized for expression in *Aspergillus niger*, synthesized and cloned to pAo62 using NcoI - BamHI restriction sites which were incorporated during gene synthesis at 5' and 3' end respectively.

The region encoding *pyrE* gene was obtained by digestion of genomic DNA from the strain *Aspergillus niger* (CBS120.49) with *SstII* restriction enzyme. For analyzing the complete DNA sequence of the genomic *SstII* fragment containing the *Aspergillus niger pyrE*, it was cloned into *SstII* digested pBLUEScript II SK (+) to obtain pBLUEpyrE. The *SstII* fragment for transformation to *Aspergillus niger* agg. (strain ASNpyrE#8) was derived from pBLUEpyrE by digestion with *SstII*. The pAo62 vector used for cloning of *lip3-prepro* gene contains the promoter and the terminator sequence of *amyA* gene from *Aspergillus oryzae*. The complete fragment *pamyA-lip3-prepro-tamyA* was extracted from the plasmid pAo62-*lip3-prepro* and co-transformed with the *pyrE* gene to the host *Aspergillus niger* agg. (strain ASNpyrE#8).

### Recipient organism

The recipient host strain *Aspergillus niger* agg. (strain ASNpyrE#8) was derived from *Aspergillus niger* agg. (strain ANSC) by spontaneous mutation in the *pyrE* gene. The mutants resistant to 5- Fluoro orotic acid were selected. The uridine/uracil auxotrophy was complemented by transforming the *pyr* marker genes. Clones transformed with the *pyrE* marker resulted in surviving colonies, indicating that these mutants were *pyrE* mutants. The *pyrE* mutant phenotype was complemented by transforming the linear DNA fragment containing the *pyrE* promoter-*pyrE*gene-*pyrE*terminator to *Aspergillus niger* agg. (strain ASNpyrE#8).

### Production strain

Two linear DNA fragments: (i) Marker gene - The 5164 bp *SstII* digested and electrophoretically purified fragment from genomic DNA of *Aspergillus niger* (CBS120.49). The *SstII* fragment was cloned to pBlueScript II SK (+) to obtain pBLUEpyrE. The *SstII* fragment for transformation to *Aspergillus niger* agg. (strain ASNpyrE#8) was derived from pBLUEpyrE by digestion with *SstII*, and (ii) Gene of interest - The 2801 bp *NotI* digested and electrophoretically purified fragment from the plasmid pAo62-*lip3-prepro* (5680 bp). This fragment includes the *Rhizopus* lipase gene with *amyA* promoter and *amyA* terminator, were transformed into the host strain *Aspergillus niger* agg. (strain ASNpyrE#8) for generating recombinant *Aspergillus niger* agg. (strain FL100SC) producing *Rhizopus* lipase enzyme. The genus *Aspergillus* includes a set of fungi that are generally considered asexual, although perfect forms (forms that reproduce sexually) have been found. Aspergilli are ubiquitous in nature. These fungi are most commonly found in mesophilic environments such as decaying vegetation or soil and plants (Schuster E. et al. 2002).

The *Aspergillus* section *Nigri* includes more than 20 taxa that produce a black pigment. Of those, several belong to the *Aspergillus niger* “aggregate” (Perrone et al, 2011), which includes, *inter alia*, *Aspergillus acidus*, *Aspergillus awamori*, *Aspergillus brasiliensis*, *Aspergillus niger* and *Aspergillus tubingensis*, all of which are morphologically indistinguishable (Samson et al, 2006, and 2007). Members of this section are often called black aspergilli or simply ‘*Aspergillus niger*’ without regard to morphological or biochemical characteristics. This creates ambiguity in attributing research-findings to a particular species of *Aspergillus* section *Nigri*.

Pertinent to note is that the same isolate has been preserved in culture collections under different species names (Abarca et al, 2004) creating multiple synonyms. This can create an ambiguous-identification (Alastruey-Izquierdo et al, 2012). Efforts are on to distinguish the above species employing a polyphasic approach including: (i) sequence analysis of parts of the  $\beta$ -tubulin and calmodulin genes and the ITS region, (ii) macro- and micromorphological analyses and (iii) examination of extrolite profiles (Frisvad et al, 2011).

Based on morphology, the parental strain of the production microorganism used for the producing the lipase was identified as *Aspergillus niger* (strain ANSC). Subsequently, the parental strain was sent to two leading mycology centres for identification confirmation, viz.: Centraalbureau voor Schimmelcultures (CBS) and Centre for Agriculture and Biosciences International (CABI) The strain ANSC was characterized using molecular methods. (Refer [Annex H2](#))

- Centraalbureau voor Schimmelcultures (CBS):

At CBS, the parental *Aspergillus niger* (strain ANSC) was identified as *Aspergillus* Section *Nigri* species. Phenotypic analysis of ANSC03131 *Aspergillus* section *Nigri* strain showed typical structures for an isolate that belongs to the *Aspergillus* section *Nigri*. Analysis of the sequence data (part of the  $\beta$ -tubulin gene and part of the Calmodulin gene amplified and sequenced from gDNA) showed that the ANSC03131 strain shared sequences with isolates of *Aspergillus acidus*, Kozak (redesignated as *Aspergillus luchuensis* by Hong et al, 2013).

Further, based on bioinformatic analysis of sequence data [partial fragment of  $\beta$ -tubulin (BT) gene and partial fragment of calmodulin (CMD) gene], the production strain FL100SC shared sequences with isolates of *Aspergillus luchuensis* (earlier known as *Aspergillus acidus* Kozak.), as determined by CBS.

- Centre for Agriculture and Biosciences International (CABI):

At CABI, the multiple unlinked genes Internal Transcribed Spacer (ITS) and Calmodulin were amplified and sequenced from gDNA of parental strain ANSC. The concatenated genes showed closeness to *Aspergillus tubingensis* and *Aspergillus acidus*. Based on these observations, CABI concluded the parental strain identified, vide CABI reference number Y12/13/H64 IMI 502637, be designated as *Aspergillus niger* agg.

Importantly, the parental strain ANSC, and production strain FL100SC were tested by CBS for mycotoxin production, and were shown not to produce any mycotoxins, establishing the safe nature of the parental and the production strains. Here, it is worthwhile noting that Hong et al, 2013, reported that extrolite analysis of strains of

*Aspergillus luchuensis* showed that they do not produce mycotoxins and therefore can be considered safe for food and beverage fermentations. Further, the strains have also been analyzed for its potential to produce ochratoxins using techniques such as PCR-RFLP methods and are considered as non ochratoxigenic fungus. (Saroj et al, 2016).

It is of relevance to note that enzymes which are approved in France and / or Denmark, and produced by *Aspergillus* species, are covered by a broad species description of *Aspergillus niger*, van Tieghem (1867). These include *Aspergillus awamori*, *Aspergillus ficuum*, *Aspergillus foetidus*, *Aspergillus phoenicis*, *Aspergillus pulverulentus*, *Aspergillus tubingensis*, *Aspergillus inuii*, *Aspergillus usamii*, *Aspergillus japonicus*, *Aspergillus saitoi*, *Aspergillus acidus*, *Aspergillus aculeatus*, and *Aspergillus niger*. (Health Canada, 2014).

The EFSA document on “Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms” (EFSA Journal; 587; 1-16, 2007) states, *inter alia*:

... The taxonomy of the section *Nigri* (the black *Aspergilli*) is not fully resolved as the number of accepted species depends on the methodology used... In general *Aspergillus niger*, **sensu lato**, has a long history of apparent safe use in biotechnology....

The ‘Inventory of Substances used as Processing Aids (IPA)’ prepared by the Codex Alimentarius Commission (Food Codex) and Clause 17 of FSANZ Standard 1.3.3 note that *Aspergillus niger* covers strains known under the names: *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus ficuum*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus phoenicis*, *Aspergillus saitoi*, *Aspergillus usamii* and *Aspergillus tubingensis*.

Likewise, JECFA and Health Canada use the term *Aspergillus niger*, *var.*, rather than *Aspergillus niger*, *sensu stricto*, in order to account for the ambiguity in the *Aspergillus niger* group nomenclature, and to include various subspecies of *Aspergillus niger* group.

In the light of the above facts, the production strain is designated as *Aspergillus niger* agg. (strain FL100SC) for uniformity and clarity of presentation.

### 2.2.3 GENETIC MODIFICATION

The genetic modification for generation of *Aspergillus niger* agg. (strain FL100SC) producing *Rhizopus* lipase enzyme consisted of three steps:

- Generation of uridine/uracil auxotroph host strain by spontaneous mutation [*Aspergillus niger* agg. (strain ASNpyrE#8)]
- Generation of fragments for transformation to *Aspergillus niger* agg. (strain ASNpyrE#8)
- Transformation of fragments and post transformation analysis for *Rhizopus* lipase activity and gene copy number.

Briefly, recombinant *Aspergillus niger* agg. (strain FL100SC) was generated as detailed below:



### Marker Gene

Step 1: The host strain *Aspergillus niger* agg. (strain ASNpyrE#8) was generated by spontaneous mutation as described above.

Step 2: Genomic DNA of *Aspergillus niger* (CBS 120.49) was digested by SstII restriction enzyme. The SstII fragment obtained by digestion of *Aspergillus niger* (CBS 120.49) was cloned to pBlueScript II SK (+) to generate pBLUEpyrE

Step 3: The *SstII* fragment containing the *pyrE* gene was extracted from pBLUEpyrE for transformation to *Aspergillus niger* agg. (strain ASNpyrE#8)

### Expression fragment for lip3 gene

Step 1: The *lip3-prepro* gene expression fragment was synthesized to facilitate cloning into expression vector pAo62.

Step 2: Synthesized *lip3-prepro* gene was cloned to pGA1

Step 3: Lip3-prepro gene was further sub-cloned from pGA1-lip3-prepro to pAo62 using restriction sites NcoI and BamHI to generate pAo62-lip3-prepro

Step 4: Two NotI sites were used for extracting the *pamyA-lip3-prepro-tamyA* construct for use in transformation

Step 5: The marker gene (*pyrE*) fragment from step 3 and expression fragment from step 4 were co-transformed to *Aspergillus niger* agg. (strain ASNpyrE#8)

Step 6: The transformants were plated on mineral media without uridine/uracil, checked for lipase activity and gene copy number.

## **2.2.4 INFORMATION ON GENETIC STABILITY**

The strategy for generating the mutant *Aspergillus niger* agg. (strain FL100SC) utilized different vectors for cloning of the marker gene and the expression fragment containing the *pamyA-lip3-prepro-tamyA*. Only the *SstII* digested and electrophoretically purified fragment for the marker gene *pyrE* and the *NotI* digested and electrophoretically purified fragment of the expression fragment (*pamyA-lip3-prepro-tamyA*) was transformed to *Aspergillus niger* agg. (strain ASNpyrE#8). Hence, there is no possibility for the presence of any vector sequence in the recombinant strain. Further, the same was also confirmed by stripping the Southern blot of gene copy number analysis and re-hybridisation with a probe specific for the vector backbone. No vector sequence was detected in any of the samples analyzed. Gene copy number of the expression fragment was determined by Southern analysis, 3 full tandem copies and one additional copy were found to be integrated at 2 sites in the genomic DNA of the host strain. Hence, total of 4 copies of *lip3-prepro* gene were found to be present in *Aspergillus niger* agg. (strain FL100SC).

The stability of the GMM was demonstrated using genetic fingerprinting technique Random Amplified Polymorphic DNA (RAPD). Two primers OPA 01 and OPA 09 were selected based on the distinct profile for determining the stability of recombinant *Aspergillus niger* agg. (strain FL100SC) during the production of *Rhizopus* lipase. Three independent batches

were analyzed and no deviation was observed in the RAPD fingerprint profile for any of the batches. The RAPD fingerprint profile of three batches was found to be similar to recombinant *Aspergillus niger* agg. (strain FL100SC) from the master cell bank. This indicates stability of cells which implies there is no genetic rearrangement in the genomic DNA of the strain during any of the process. For identification details of strain FL100SC refer [Annex H1](#).

### 2.2.5 **GOOD INDUSTRIAL LARGE SCALE PRACTICE (GILSP)**

The source material used for the production of lipase covered in this dossier is genetically modified *Aspergillus niger* agg. (strain FL100SC). The organism is deposited with the American Type Culture Collection (ATCC), and the safe deposit number is SD-6846. The organism is derived from the parental strain *Aspergillus niger* (strain ASNSC). Studies conducted by CBS on the parental strain *Aspergillus niger* (strain ASNSC) and the production strain *Aspergillus niger* agg. (strain FL100SC), showed that neither of them produce mycotoxins to detectable levels. ([Annexes H1](#) , [H2](#)).

Further, *Rhizopus* lipase and *Aspergillus niger* strains have a long history of safe use in industrial-scale enzyme production and can be considered as safe production organisms for enzymes used in food / feed processing as well as numerous other industrial applications. *Rhizopus* lipase is approved by Health Canada, Brazil Food Authority, Légifrance, GB list of China and Food standards of Australia and New Zealand. Also, FDA had no questions regarding the conclusion that *Rhizopus* lipase enzyme preparation is GRAS under the intended conditions of use. (GRN No. 000216)

During recent years, genetic engineering techniques have been used to improve the industrial production strains of *Aspergillus niger* and considerable experience on the safe use of recombinant *Aspergillus niger* strains at industrial scale has accumulated. FAO/WHO experts have repeatedly reviewed and accepted enzyme preparations from *Aspergillus niger*, including the organism itself (FAO/WHO 1972, 1978, 1981, 1987, 1990), listing them with an Acceptable Daily Intake (ADI) of ‘not specified’.

The EFSA document on “Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms” states, *inter alia*, that *Aspergillus* section *Nigri* are used in biotechnology, for the production of enzymes, and that *Aspergillus niger*, *sensu lato*, has a long history of apparent safe use in biotechnology.

The FDA in the United States has accepted numerous enzymes for food use: in the early 1960s the FDA issued opinion letters recognizing, *inter alia*, that lipase from *Aspergillus niger* can be ‘generally regarded as safe’ (GRAS) under the condition that non-pathogenic and non-toxicogenic strains and current good manufacturing practices be used in production (Schuster et al 2002). In the Food Standards Code-1.3.3-processing aids of FSANZ, lipase from *Aspergillus niger* is listed as safe for use in food. Further, secondary metabolites are of no safety concern in fermentation products derived from *Aspergillus niger*.

Additionally, *Rhizopus* lipase covered in this dossier and produced by *Aspergillus niger* agg. (strain FL100SC), does not contain mycotoxins or heavy metals above specified detection levels. This was further substantiated by the lack of toxicity, when the food enzyme object

was tested in standard *in vitro* (OECD 471 - Ames' test and OECD 473 - chromosomal aberrations studies) and *in vivo* (OECD 408 – Repeated dose 90-day oral toxicity studies) model systems.

The lipase covered in this dossier and produced by *Aspergillus niger* agg. (strain FL100SC) is manufactured according to current GMP, and the principles of HACCP.

Along the whole manufacturing process, in order to comply with current GMP and HACCP principles for food production, the following potential hazards in food enzyme production are taken into account:

- Identity and purity of the producing microorganism: the microorganism that is effectively fermented, must be the one selected and evaluated for the production of the food enzyme.
- Microbiological hygiene: ubiquitous microorganisms must not contaminate the process.
- Chemical contaminants: raw materials used in the manufacturing process must be suitable for the intended purpose and comply with the specifications.

As a result, *Aspergillus niger* agg. strain FL100SC can be used under the lowest containment level at large scale, GILSP, as defined by OECD (1992) [refer [Annex G](#)].

#### **2.2.6 ABSENCE OF THE PRODUCTION ORGANISM IN THE PRODUCT**

The lipase covered in this dossier did not contain detectable amount of full length recombinant DNA as determined by the absence of amplicon for full length recombinant gene of interest. Likewise, when the lipase covered in this dossier was analyzed for the presence/absence of the production microorganism, the test lipase samples and negative controls did not show any fungal colony on petri plates, as opposed to positive control, which showed growth. These results show that the product is free of the production microorganism (GMM) and recombinant DNA.

#### **2.2.7 ABSENCE OF TRANSFERABLE RECOMBINANT DNA SEQUENCES IN THE ENZYME PREPARATION**

As described above, only the digested and electrophoretically purified fragment for the marker gene (*pyrE* and the expression fragment (*pamyA-lip3-prepro-tamyA*) was transformed to *Aspergillus niger* agg. (strain ASNpyrE#8). Hence, there is no possibility for the presence of any vector sequence in the recombinant strain. Further, the same was also confirmed by stripping the Southern blot of gene copy number analysis and re-hybridisation with a probe specific for the vector backbone. No vector sequence was detected in any of the samples analyzed. Gene copy number of the expression fragment was determined by Southern analysis, 3 full tandem copies and one additional copy were found to be integrated at 2 sites in the genomic DNA of the host strain. Hence, total of 4 copies of *lip3-prepro* gene were found to be present in *Aspergillus niger* agg. (strain FL100SC).

#### **2.2.8 ABSENCE OF ANTIBIOTIC GENES AND TOXIC COMPOUNDS**

No functional antibiotic resistance genes were introduced into the host during GMM production and hence the strain is free from any antibiotic gene (Refer [Section 2.2.4](#)). As specified earlier, the lipase covered in this dossier does not contain mycotoxins or heavy

metals above specified detection levels. This was further substantiated by the lack of toxicity, when the food enzyme object was tested in standard *in vitro* (OECD 471 - Ames' test and OECD 473 - chromosomal aberrations studies) and *in vivo* (OECD 408 – Repeated dose 90-day oral toxicity studies) model system.

### 2.2.9 **SECONDARY METABOLITES**

Most industrial strains of *Aspergillus niger* are from safe strain lineages that have been repeatedly tested according to the criteria laid out in the Pariza & Johnson publication (Pariza & Johnson, 2001). [Refer [Section 6.2.3](#)]

*Aspergillus niger* strains have been safely used for decades to produce citric acid as well as a wide variety of food enzymes. Since it was recognized that some strains of fungal origin, such as *Aspergillus niger*, might be able to produce certain secondary metabolites of potential safety concern, JECFA recommended testing food enzymes derived from fungal origin for the presence of the secondary metabolites that could theoretically be produced by the species, i.e. ochratoxin A and fumonisins (Frisvad et al., 2011)

The parental strain ASNSC, and production strain FL100SC were tested by CBS for mycotoxin production, and were shown not to produce any mycotoxins, establishing the safe nature of the parental and the production strains. Further, the production strain has also been analyzed for its potential to produce ochratoxins using techniques such as PCR-RFLP methods and is considered as non ochratoxigenic fungus. (Saroj et al, 2016)

Likewise, the toxicological studies (Refer [Section 6.4](#) and [Annex K](#)) performed on the food enzyme, lipase produced by *Aspergillus niger* agg. (strain FL100SC), show that there is no cause for any toxicological concern.

## 2.3 MANUFACTURING PROCESS

### 2.3.1 OVERVIEW

The lipase described in this dossier is manufactured in accordance with current Good Manufacturing Practice for Food (cGMP) and the principles of Hazard Analysis of Critical Control Points (HACCP). Refer [Annex F](#) for details.

It is in the interest of the enzyme industry itself to produce high quality, functional and safe food enzymes in an economic efficient way. Consequently, a strictly controlled process is a prerequisite of food enzyme production.

In the following Sections, the key steps of the fermentation ([Section 2.3.2](#)), recovery ([Section 2.3.3](#)) and formulation and packaging ([Section 2.3.4](#)) of the food enzyme lipase is described. These sections include a description of process parameters, raw materials and other chemical substances as well as materials and equipment used. [Section 2.3.4.1](#) includes the enzyme immobilization procedure. Finally, [Section 2.3.6](#) describes the measures taken to comply with current GMP and HACCP.

It should be noted that the fermentation process of microbial food enzymes is substantially equivalent across the world. This is also true for the recovery process: in a vast majority of cases, the enzyme protein in question is only partially separated from the other organic material (TOS, refer [Section 2.4.1](#)) present in the food enzyme

### 2.3.2 FERMENTATION

The production of food enzymes from microbial sources follows the process involving fermentation as described below. Fermentation is a well-known process that occurs in food and has been used for the production of food enzymes for decades. This section first describes the raw materials used in fermentation, followed by a description of the different fermentation process steps:

- Inoculum
- Seed fermentation
- Main fermentation

Liquid state/ submerged fermentation is used to produce the *Rhizopus* lipase from *Aspergillus niger* (strain FL100SC). The description below covers the various processes involved, as they can be considered substantially equivalent. All parameters are within similar ranges.

The typical batch size ranges from 18000 L to 55000 L and depending on the market demand, the frequency of production of the food enzyme varies from once a week, to once in two years

#### 2.3.2.1 Raw materials

Materials used in the fermentation process (inoculum, seed and main fermentation) are:

- Potable water
- A carbon source
- A nitrogen source
- Salts
- Vitamins (as a part of complex fermentation materials)



- pH adjustment agents
- Foam control agents

### 2.3.2.2 Inoculum

A suspension of a pure culture of *Aspergillus niger* agg. (strain FL100SC) is aseptically transferred to an inoculum flask containing fermentation medium.

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

### 2.3.2.3 Seed Fermentation

The inoculum is aseptically transferred to the seed fermentor containing fermentation medium. When a sufficient amount of biomass has developed (after about 25-35 h), the content of the seed fermentor is used for inoculation of the main fermentation.

### 2.3.2.4 Main fermentation

Biosynthesis of the enzyme protein by the production organism occurs during the main fermentation.

Liquid-state fermentation

The fermentation in the main fermentor is operated as a fed batch fermentation, wherein the content of the seed fermentor is aseptically transferred to the main fermentor containing fermentation medium. The fermentation process is continued for a predetermined time or until laboratory test data show that the desired enzyme production has been obtained or that the rate of enzyme production has decreased below a predetermined production rate. When these conditions are met, usually after appropriate time interval and depending on the batch size, the fermentation is completed.

## 2.3.3 RECOVERY

The purpose of the recovery process is to separate the fermentation broth into biomass and fermentation medium containing the desired enzyme protein.

During fermentation, the enzyme protein lipase is secreted by the producing microorganism *Aspergillus niger* agg. (strain FL100SC) into the fermentation medium. During recovery, the enzyme-containing fermentation medium is separated from the biomass.

This section first describes the materials used during recovery (downstream processing), followed by a description of the different recovery process steps:

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

The nature, number and sequence of the different types of unit operations described below may vary, depending on the specific enzyme production plant.

### **2.3.3.1 Materials**

Materials used, if necessary, during recovery of the food enzyme include:

- Filter aids To facilitate the solid/ liquid separation
- pH adjustment agents To facilitate the solid/ liquid separation and/or stabilize the enzyme viz. Sodium hydroxide
- Stabilizer To stabilize enzyme concentration. viz. Glycerine, Maltodextrin etc.

Potable water can also be used in addition to the above mentioned materials during recovery.

### **2.3.3.2 Primary Separation**

Filter aids are added at a controlled pH to enable the separation of the biomass from liquid. The fermentation broth is pressed through a horizontal filter press (a series of filter chambers) leaving the solids on the surface of the filters. If necessary the filtering cycle is repeated. The filter aids and the biomass are then removed from the filtrate. The primary separation is performed at defined pH and temperature ranges in order to minimize loss of enzyme activity.

### **2.3.3.3 Concentration**

The enzyme is concentrated by ultrafiltration and diafiltration to reach the desired enzyme activity. The temperature and pH are controlled during the concentration step.

### **2.3.3.4 Pre-filtration and micro-filtration (Germ filtration)**

A filtration step on a dedicated micro (germ) filtration media is applied to ensure the removal of the production strain cells and insoluble substrate components from the fermentation. A pre-filtration step is included when needed. The concentrated enzyme liquid obtained after microfiltration, if required, is stabilized using suitable stabilizer such as glycerol.

### **2.3.3.5 Spray Drying**

The concentrated enzyme solution is spray dried in presence of food grade stabilizer(s) to obtain unformulated concentrate.

## **2.3.4 FORMULATION AND PACKAGING**

Food enzymes can be sold as powder, liquid or immobilized preparations, depending on the final intended application.

- For the manufacturing of dry enzyme preparation, the spray-dried unformulated concentrate [Not less than (NLT) 5,00,000 FIP U/g] is formulated to the declared activity with food grade formulating agents such as maltodextrin.

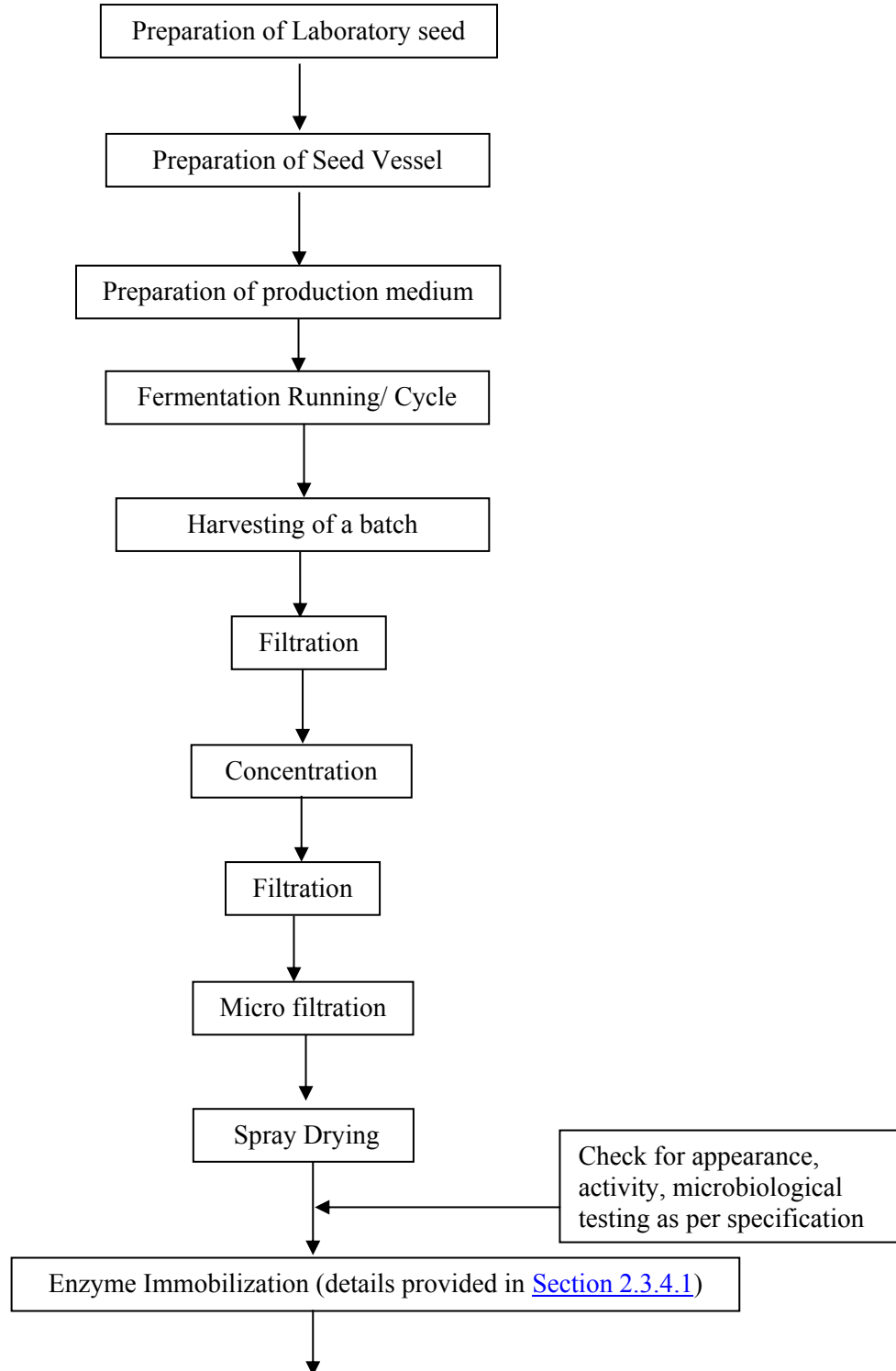
The enzyme maybe immobilized on appropriate carrier as mentioned in [Section 2.3.4.1](#) and [Annex J](#)

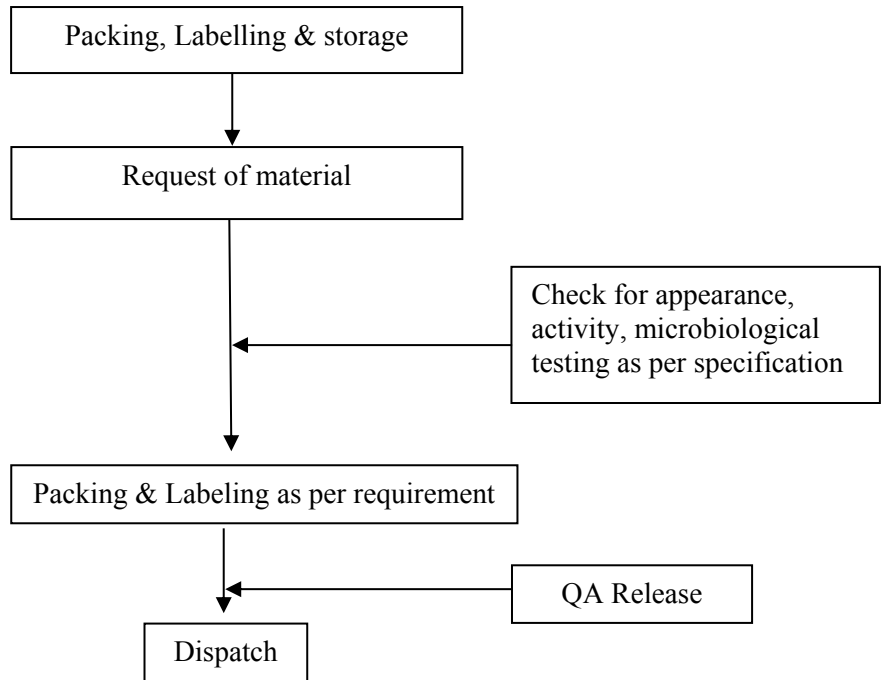
The final formulated product is called ‘food enzyme preparation’.

The food enzyme preparation is tested by Quality Control for all quality related aspects, like expected enzyme activity and the general JECFA Specification for Food Enzyme Preparations, and released by Quality Assurance. The final product is packed in suitable food packaging material before storage. Refer [Section 2.4](#) of this document for analytical data on a

statistically relevant number of manufactured batches. Warehousing and transportation are performed according to specified conditions mentioned on the accordant product label for food enzyme preparations.

The general production flow-chart of immobilized *Rhizopus* lipase is provided below:





### 2.3.4.1 Immobilization

#### PROCEDURE

The food enzyme lipase is immobilized.

Details of immobilization (enzyme support materials and immobilizing agents, etc..)

**Enzyme:** *Rhizopus* Lipase produced from genetically modified *Aspergillus niger* agg. (strain FL100SC)

**Carrier:** Suitable carrier<sup>1</sup>

#### Procedure:

The powder product is dissolved in water and the pH is adjusted. This liquid is added to carrier which has been prewashed 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.

#### INFORMATION ON POTENTIAL LEAKAGE OF CARRIERS, IMMOBILISATION AGENTS AND ACTIVE ENZYMES INTO THE FOOD

##### (i) Leakage of enzyme into food – experimental proof

Method: In a model reaction, interesterification was carried out using 3.75g palm stearin, 11.25g coconut oil and 45 ml n-hexane equilibrated at 55°C. The reaction was initiated by 1.5 g of immobilized enzyme and continued at 55°C in shaker cum incubator at 200 rpm for 4h. The samples, before the addition of enzyme and 4h post the reaction were analyzed for nitrogen/ protein content by Kjeldahl's method.

Result:

Sample	Nitrogen%	Protein content (%)
Initial reaction mix (Before enzyme addition)	0.0342	0.214
With 10% enzyme loading w.r.t. substrate (Post 4h enzyme reaction)	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 of the fact that the enzyme sample, which is basically proteinaceous in nature, does not leach into food. (Please refer [Annex J](#) for details)

<sup>1</sup> The carrier resin under use is crosslinked copolymer of Methacrylate. This carrier complies with the regulations of the COUNCIL OF EUROPE COMMITTEE OF MINISTERS' Resolution ResAP(2004)3 on ion exchange and adsorbent resins is used in the processing of foodstuffs.

Appendix VII of [Annex J](#) has a list of various resins and our resin of interest is highlighted in yellow. A separate PDS detailing the information on resin of interest has been provided in Annex VII of [Annex J](#)



- (ii) Leakage of carriers/immobilization agents into food.

The immobilization support/ carrier material complies with Resolution ResAP (2004) 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 foodstuff or deterioration in the organoleptic characteristics thereof. (Refer [Annex J](#) for details)

### **2.3.5 GOOD MANUFACTURING PRACTICE AND HACCP**

#### **2.3.5.1 Potential Hazards**

In order to comply with current GMP and HACCP principles for food production, the following potential hazards in food enzyme production are taken into account:

##### **A. IDENTITY AND PURITY OF THE PRODUCING MICROORGANISM**

The assurance that the production microorganism efficiently produces the desired enzyme protein is of utmost importance to the food enzyme producer. Therefore it is essential that the identity and purity of the microorganism are controlled.

##### **B. MICROBIOLOGICAL HYGIENE**

For optimal enzyme production, it is important that hygienic conditions during the whole fermentation process are controlled. Microbial contamination would immediately result in less growth of the production organism and consequently in a low yield of the wanted enzyme protein and eventually a rejected product.

##### **C. CHEMICAL CONTAMINANTS**

It is also important that the raw materials used during fermentation are of suitable quality and do not contain contaminants which might affect the product safety of the food enzyme and/or the optimal growth of the production organism and thus enzyme yield.

#### **2.3.5.2 Control Measures**

The main measures to control the hazards identified above are:

##### **A. IDENTITY AND PURITY OF THE PRODUCING MICROORGANISM**

Production of the required enzyme protein is based on a well-defined Master Cell Bank (MCB) and Working Cell Bank (WCB). A Cell Bank is a collection of ampoules containing a pure culture. The cell line history and the production of a Cell Bank, propagation, preservation and storage is monitored and controlled. The MCB is prepared from a selected strain. The WCB is derived by sub-culturing of one or more ampoules of the MCB. A WCB is only accepted for production runs if its quality meets the required standards. This is determined by checking identity, viability, microbial purity and productivity of the WCB. The accepted WCB is used as seed material for the inoculum.

## B. MICROBIOLOGICAL HYGIENE

Measures to guarantee microbiological hygiene and prevent contamination with microorganisms ubiquitously present in the environment (water, air, raw materials) are:

- Hygienic design of equipment
- Cleaning and sterilization:
  - Validated standard cleaning and sterilization procedures of the process area and equipment
  - Sterilization of all fermentation media
  - Use of sterile air for aeration of the fermentor
- Hygienic processing:
  - Aseptical transfer of the content of the WCB ampoule, inoculum flask or seed fermentor.
  - Maintaining a positive pressure in the fermentor
- Germ filtration

## C. CHEMICAL CONTAMINANTS

It is ensured that all raw materials used in production of food enzymes are of food grade quality or have been assessed to be fit for their intended use and comply with agreed specifications.

### 2.3.5.3 In process testing and monitoring

In addition to the above mentioned control measures in-process testing and monitoring is performed to guarantee an optimal and efficient enzyme production process and a high quality product (GMP).

These in-process controls comprise:

#### A. MICROBIAL CONTROLS

Absence of significant microbial contamination is analyzed by microscopy or plate counts before inoculation of both the seed and main fermentation and at regular intervals and at critical process steps during fermentation and recovery.

#### B. MONITORING OF FERMENTATION PARAMETERS

Monitoring of fermentation parameters may include *inter alia* pH and temperature.

The measured values of these parameters are constantly monitored during the fermentation process. The values indicate whether sufficient biomass or enzyme protein has been developed and the fermentation process evolves according to plan.

Deviations from the pre-defined values lead to adjustment, ensuring an optimal and consistent process.

#### C. ENZYME ACTIVITY AND OTHER RELEVANT ANALYSES (LIKE TOTAL SOLUBLE SOLIDS)

This is monitored at regular intervals and at critical steps during the whole food enzyme production process. For ease of reference, the Hazard Analysis and Critical Control Points (HACCP) program followed at Advanced Enzyme Technologies is presented in [Annex F](#).

### 2.3.6 **QUALITY CONTROL OF FINISHED PRODUCT**

The proposed food enzyme preparation conforms to specifications established for enzyme preparations in the Food Chemicals Codex (FCC, 10th edition, 2017) and the General Specifications and Considerations for Enzyme Preparations Used in Food Processing established by the FAO/WHO Joint Expert Committee on Food Additives (JECFA, 2006).

The specification of enzyme described in [Section 2.4](#).

Test	Limits	Reference
Heavy metals	Not more than 30 mg/kg	AOAC 18 <sup>th</sup> Edn 2005 (ICP)
Lead	Not more than 5 mg/kg	AOAC 18 <sup>th</sup> Edn 2005 (ICP)
<i>Salmonella</i> sp.	Absent in 25 g of sample	Harmonized Pharmacopoeial method (BP, USP and IP)
Total coliforms	Not more than 30 per gram	Harmonized Pharmacopoeial method (EP, BP, USP, and IP)
<i>Escherichia coli</i>	Absent in 25 g of sample	Harmonized Pharmacopoeial method (EP, BP, USP, and IP)
Antimicrobial activity <sup>2</sup>	Not detected	JECFA 2003 (FNP 52, Add. 11)
Mycotoxins	No significant levels <sup>3</sup>	TNO MYC/024 (LC-MS/MS)

Note: Enzyme activity of powder lipase complies with the Food Chemical Codex (10<sup>th</sup> edition)

<sup>2</sup> Please refer [Annex E2](#)

<sup>3</sup> Refer JECFA specifications, <ftp://ftp.fao.org/docrep/fao/009/a0675e/a0675e00.pdf>, page 64: Although nonpathogenic and nontoxic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include strains capable of producing low levels of certain mycotoxins under fermentation conditions conducive to mycotoxin synthesis. Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species.

## 2.4 COMPOSITION AND SPECIFICATION

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

The TOS value is an internationally accepted method to describe the chemical composition of commercial food enzymes. The ratio between the enzyme activity and TOS is an indication of the relative purity of the enzyme.

### 2.4.1 QUANTITATIVE COMPOSITION

Relative purity of *Rhizopus* lipase from *Aspergillus niger* agg. (strain FL100SC), were measured, and the TOS values were calculated, in 3 batches after drying. The results are shown in the following Table, and the details are presented in [Section 2.4.1.1](#)

Table 2.4.1-1 Analysis of compositional variability

Batch no	0512178	0413134	011423 <sup>4</sup>	Mean enzyme activity
Ash (%)	7.89	8.97	8.14	-
Water (%)	6.63	7.45	6.95	-
TOS (%)	85.48	83.58	84.91	-
Lipase activity (FIP/g)	552,118	520,231	548,964	540437.67
FIP/mg TOS	645.90	622.43	646.52	-
One unit of enzyme activity (FIP) is defined as that quantity of a standard lipase preparation (Fungal Lipase International FIP standard) that liberates the equivalent of 1µmol of fatty acid per minute from the substrate emulsion under the described assay conditions. In case of immobilized preparation, the batch Inter esterification Unit (BIU) is employed in order to illustrate the esterifying potential of the immobilized lipase enzyme preparation. One BIU is defined as the amount of immobilized lipase that incorporates lauric acid at an initial rate of 1µmole/ minute at standard conditions of the assay.				

The standardized methods for determining the ash, dry matter content (to calculate the TOS), heavy metals, pathogens, mycotoxins and antimicrobial activity are given in [Annex A1](#).

The method, by which enzyme activity is measured, including an explanation of the Units is given in [Annex A2](#)

<sup>4</sup> The batch used for toxicity studies adheres to JECFA specifications for food enzyme(s).

### 2.4.1.1 Data on Batch-To-Batch Variability for Relevant Parameters

The compositional variability of three powder batches of *Rhizopus* lipase from *Aspergillus niger* agg. (strain FL100SC) was analyzed. Summary results for the powder batches are included in the tables below:

Table 2.4.1.1-2: Analysis of compositional variability of *Rhizopus* lipase from *Aspergillus niger* (strain FL100SC) –powder

Lot No.	Date Produced	% Ash	% LOD	TOS	U/mg TOS	Active substance (NLT 500,000 FIP U/g)
0512178	May, 2012	7.89	6.63	85.48	645.90	552,118
0413134	April, 2013	8.97	7.45	83.58	622.43	520,231
011423	January, 2014	8.14	6.95	84.91	646.52	548,964
<i>Mean</i>						540437.67
<i>SD (Standard Deviation)</i>						17570.40
<i>CV%</i>					<i>SD/mean)*100</i>	3.25
<i>Homogeneity</i>					<i>(100-CV%)</i>	96.75

Table 2.4.1.1 - 1a: Analysis of compositional variability of *Rhizopus* lipase from *Aspergillus niger* (strain FL100SC) –powder

Lot No.	Date Produced	Heavy Metal (Lead)	Pathogen (Negative by test)	Mycotoxin (Absent by test)	Antimicrobial Activity
0512178	May, 2012	< 5ppm	Complies	Complies	Absent by test
0413134	April, 2013	< 5ppm	Complies	Complies	Absent by test
011423	January, 2014	< 5ppm	Complies	Complies	Absent by test

The proof that the *Rhizopus* lipase from *Aspergillus niger* agg. (strain FL100SC) complies with these specifications is shown by the analyses on various different batches. Refer [Annex D](#) and [Annexes E1](#) and [E2](#) for analyses of heavy metals, mycotoxins and anti-microbial activity, respectively. [Annex A1](#) provides information covering microbial specifications.



## 2.4.2 SPECIFICATIONS

### Specification of *Rhizopus* Lipase powder

<b>PHYSICAL PARAMETER</b>		
Description	:	Light brown to brown colored powder; having typical fermentative odour
<b>CHEMICAL PARAMETER</b>		
Solubility	:	Soluble in water.
Heavy metal	:	Not more than 30 ppm
Lead	:	Not more than 5 ppm
Arsenic	:	Not more than 3 ppm
<b>APPLICATION PARAMETER</b>		
Lipase activity	:	Not less than 500,000 FIP U/g
<b>MICROBIOLOGICAL PARAMETER</b>		
Total Viable Count	:	Not more than 50000cfu/g
<i>Total Coliform</i>	:	Not more than 30 cfu/g
<i>Escherichia coli</i> /25g	:	Negative by test
<i>Salmonella</i> spp./25 g	:	Negative by test
Antimicrobial activity	:	Absent by test.
Mycotoxins	:	Absent by test.

### Specification of immobilized *Rhizopus* Lipase

<b>PHYSICAL PARAMETER</b>		
Description	:	Off white to cream colored granules
Loss on Drying	:	Not more than 7%
<b>CHEMICAL PARAMETER</b>		
Heavy metal	:	Not more than 30 ppm
Lead	:	Not more than 5 ppm
Arsenic	:	Not more than 3 ppm
<b>APPLICATION PARAMETER</b>		
Lipase activity	:	Not less than 200 BIU/g
<b>MICROBIOLOGICAL PARAMETER</b>		
Total Viable Count	:	Not more than 50000cfu/g
<i>Total Coliform</i>	:	Not more than 30 cfu/g
<i>Escherichia coli</i> /25g	:	Negative by test
<i>Salmonella</i> spp./25 g	:	Negative by test
Antimicrobial activity	:	Absent by test.
Mycotoxins	:	Absent by test.

## 2.5 TECHNICAL EFFECT

### 2.5.1 **MODE OF ACTION**

Lipase catalyzes the hydrolysis of carboxylic ester bonds in glycerides and producing free fatty acids, diacylglycerols, monoacylglycerols and glycerols. In addition to its natural action of hydrolysis, lipase catalyzes the esterification of fatty acids and alcohols or rearranges fatty acids in glycerides under micro-aqueous condition. (Houde et. al., 2004; Aravindan et al. 2007)

### 2.5.2 **USES**

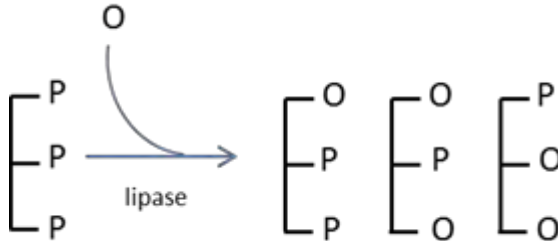
Fats and oils are important constituents of foods. The nutritional, sensory value and the physical properties of a triglyceride are greatly influenced by factors such as the position of the fatty acid in the glycerol backbone, the chain length of the fatty acid, and its degree of unsaturation. These characteristics/properties maybe enhanced by hydrogenation, fractionation, blending, and esterification of fats and oils. Chemical esterification and hydrogenation require harsh processing, often resulting in high levels of waste products – and hydrogenation also generates trans-fatty acids. Lipases allow modification of lipids by the interesterification/ rearrangement of fatty acid chains in the glyceride and replacing one or more of the fatty acids with new ones. This improves the physical and nutritional properties of glyceride products. Thus, a relatively inexpensive and desirable lipid can be modified to a higher value fat. (R. Sharma et al. 2001)

The method for the modification and improvement of edible fats by interesterification is a well-known procedure in the fat industry (Macrae et al, 1983; Houde et. al., 2004; de Paula et al., 2010, Bornscheuer et. al, 2013; Ferreira-Dias et al, 2013). FDA has accepted this technology in approving the GRAS notifications for several modified fats (“High 2-Palmitic Vegetable Oil/Betapol Structured Triglycerides”, GRN 000131 (Loders Croklaan, 2003) and GRN 000192 (Enzymotec, Ltd. 2006, updated 2012); “Tailored Triglycerides Containing Approximately 12 Percent Medium-Chain Fatty Acids”, GRN 000217 (Nisshin OilliO Group USA, Inc., 2006)] and for using lipases to make modified fats, e.g., 21 CFR 184.1420 “Lipase Enzyme Preparation from *Rhizopus niveus* Used in the Interesterification of Fats and Oils”; “Lipase from *Candida rugosa*”, GRN 000081 (Amano Enzyme Inc., 2001); “Lipase enzyme preparation from *Aspergillus oryzae*”, GRN 000113 (Enzyme Technical Association, 2002); and “Lipase enzyme preparation from *Rhizopus oryzae*”, GRN 000216 (Amano Enzyme Inc.,2006).

The products intended to be manufactured using *Rhizopus* lipase include, Human milk fat substitute and Cocoa butter substitute.

**Human milk fat substitutes:** Triglycerides in human milk have around 70% of the palmitic acid located at the sn-2 (middle) position. Currently used triglycerides in infant formulas contain palmitic acid predominantly in the sn-1 and sn-3 positions, which affects the absorption of the fatty acids, and reduces nutrient bioavailability (GRN 000113, Loders Croklaan, 2003). Hence, the structured lipids produced with the action of a 1,3 specific lipase yields a lipid closer to human milk fat. The predominant triglyceride in these lipids is 1,3-dioleoyl 2-palmitoyl triglyceride.

The first step in the manufacturing process involves an interesterification reaction (technically best defined as acidolysis) between palm stearin, a palm oil triglyceride fraction rich in palmitate (PPP), and the added food grade oleic acid using a safe and suitable 1,3-specific lipase for fat interesterification in foods.



*Fig: Selective interesterification of the main triglyceride of palm stearine (PPP) with the *Rhizopus* lipase to obtain a mixture of triglycerides enriched in the triglycerides PPO, OPO and POO.*

The second step in the manufacturing process for the formation of beta-palmitin involves the removal of the excess free fatty acids (primarily O) by distillation and the physical fractionation of the product of interesterification to obtain the beta-palmitin, a fraction further enriched in OPO. Refining consists of the traditional vegetable oil refining, including bleaching and deodorisation. All processing chemicals used in the manufacture of beta-palmitin are appropriate for food-use.

The process flow of human milk fat substitutes presented below shows the typical application of the food enzyme and shows the conditions under which the food enzyme is used

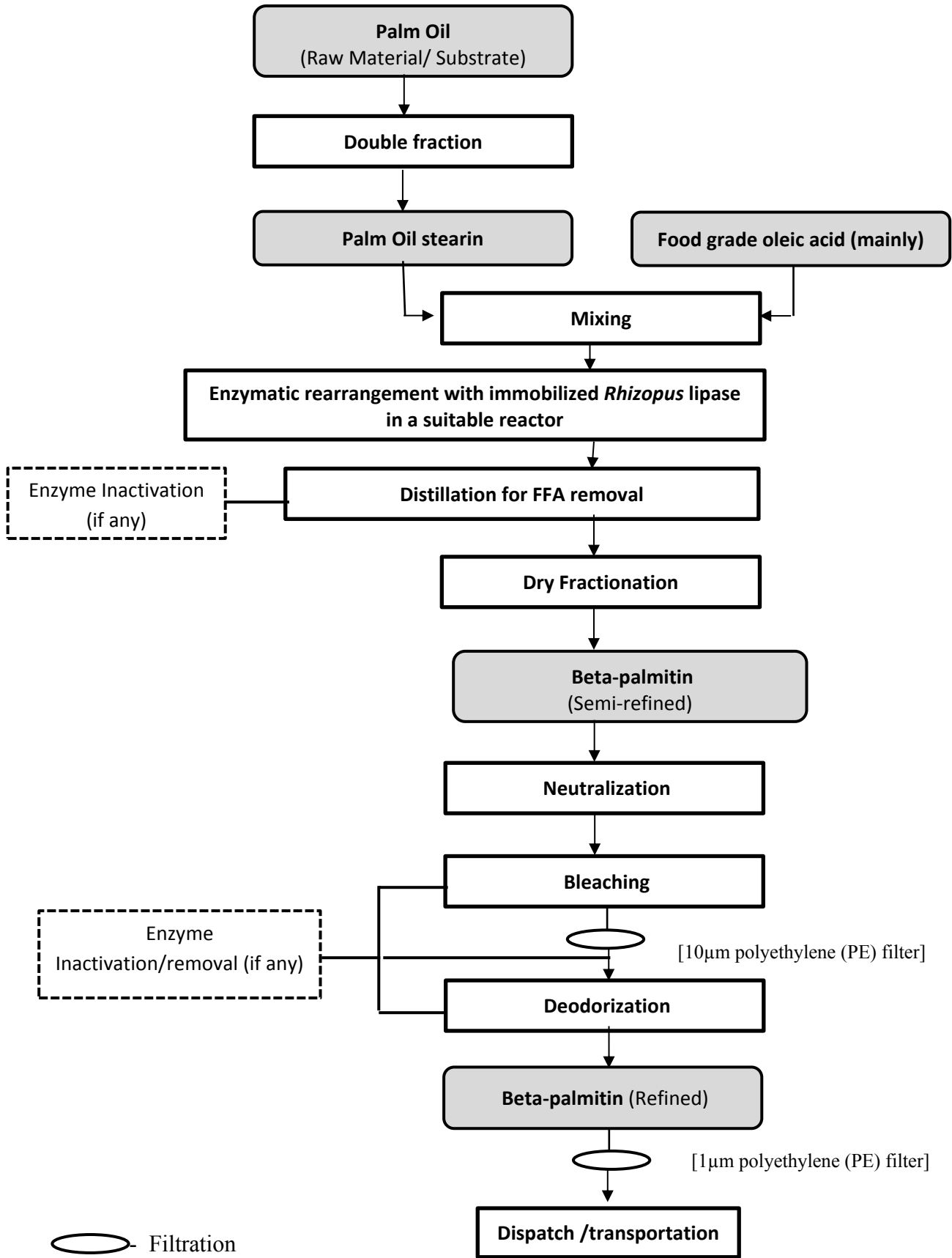
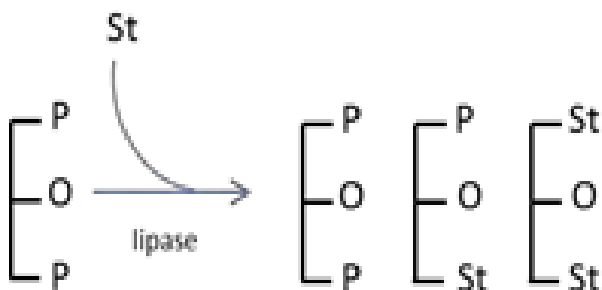


Figure 2.5.2 -1: Flow-chart - Use and fate of immobilized lipase in Cocoa butter equivalent

**Cocoa butter Equivalents (eCBE):** A high demand for chocolate and escalating cocoa butter prices has prompted a considerable amount of research focusing on cheaper and suitable alternatives to cocoa butter (Lipp & Anklam, 1998). The triglycerides 1, 3-dipalmitoyl-2-oleylglycerol (POP), 1-palmitoyl-2-oleyl-3-stearoylglycerol(POSt) and 1, 3-stearoyl-2-oleylglycerol (StOSt) are the predominant components of Cocoa butter. These particular triglycerides are largely responsible for providing chocolates with their characteristic textural and sensory properties. Cocoa butter equivalents' (CBE) closely matching this composition is desired.

The first step in the manufacturing process for the making of eCBE involves an interesterification reaction between PMF (Palm Mid Fraction), a palm oil triglyceride fraction rich in palmitate (POP), and the added food grade stearic acid using a safe and suitable 1,3-specific lipase.



*Fig: Selective interesterification of the main triglyceride of PMF (rich in POP) with the lipase to obtain interesterified PMF, a mixture of triglycerides enriched in the triglycerides POP, POSt and StOSt. The palmitic acid residues (P) on the triglyceride POP are replaced by stearic acid (St) residues at the positions sn-1 and sn-3*

The second step in the manufacturing process for the formation of eCBE involves the removal of the excess free fatty acids (primarily P) by distillation and the (solvent or dry) fractionation of the product of interesterification to obtain an olein fraction of the interesterified PMF further enriched in POP, POSt and StOSt

In the third step a lipase treatment (Lipase having activity only on di and mono glycerides) is required to fully hydrolyze possible diglycerides and monoglycerides present in the mixture since small amounts of diglycerides and monoglycerides can heavily affect the crystallization properties of the cocoa butter equivalent (Smith et al, 2011). It is pertinent to note that the lipase treatment mentioned in this paragraph is mutually exclusive to the technological function performed by *Rhizopus* lipase of the current dossier.

Refining consists of the traditional vegetable oil refining, including bleaching and deodorisation. All processing chemicals used in the manufacture of eCBE are appropriate for food-use.



The process flow of Cocoa butter substitutes/ Equivalents presented below shows the typical application of the food enzyme and shows the conditions under which the food enzyme is used.

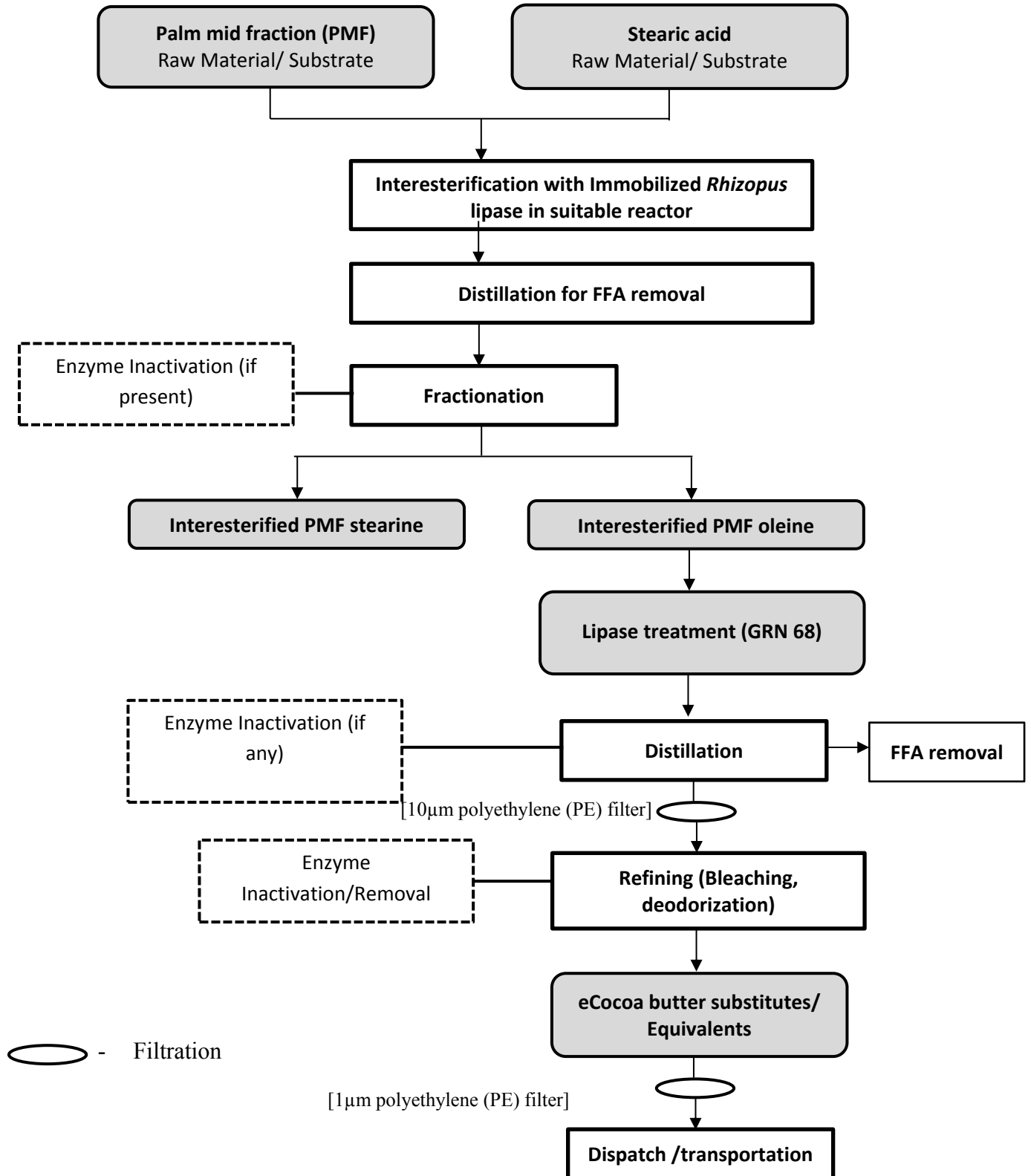


Figure 2.5.2 -2: Flow-chart - Use and fate of immobilized lipase in Cocoa butter equivalents

### 2.5.3 **USE LEVELS**

In principle, the enzymatic conversion of glycerides present in fats & oil with the help of lipase can be used in the processing of all food raw materials which naturally contain these substrates.

The lipase covered in this dossier is typically used in modification / esterification of lipids, more specifically for the preparation of human milk substitutes and cocoa butter equivalents.

Studies conducted in house indicate that the residues of denatured *Rhizopus* lipase, if present in the final triglyceride will not exceed 100 parts per million (ppm). The weight percent of *Rhizopus* lipase immobilized on the carrier is between 5-10%. Typically, 0.3-1kg of immobilized *Rhizopus* lipase granules is used per ton of oil/fat.

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

In principle, the enzymatic conversion of glycerides with the help of lipase can be used in the modification/ esterification of lipids which naturally contain glycerides. In these processes, the lipase is used as a processing aid in food manufacturing and is not added directly to final foodstuffs.

Lipase performs its technological function during food processing and does not perform any technological function in the final food. The reasons why the enzyme does not perform any technological function in the final food can be due to a combination of various factors, depending on the application and the process conditions used by the individual food producer. These factors include denaturation of the enzyme during processing, depletion of the substrate, lack of water activity, unconducive pH, etc..

Based on the conditions of use described in [Section 2.5.2](#) and the activity of the lipase under such conditions (refer [Section 2.1.3](#)), it can be concluded that the lipase does not perform any technological function in the final food.

The fate of the lipase in various processes, are explained in more detail below.

#### **Human milk fat substitutes:**

Immobilized Lipase is insoluble and physically stable in oil; furthermore, after coming in contact with the enzyme preparation, the oil will be subjected to oil refining methods which include processing steps such as filtration, bleaching and deodorization which would remove any potential enzyme residue and carrier material if present. It is important to note that during refining, before deodorization, esterified lipid (beta palmitin) is filtered with a 10 µm polyethylene (PE) filter and further prior to loading in trucks, beta palmitin is again filtered using a 1µm PE filter to make sure that no particles (e.g. immobilized enzyme, unbound resin) are present in the oil.

**Cocoa butter substitutes/ Equivalentents:**

Immobilized lipase is insoluble in oil; furthermore, after coming in contact with the enzyme preparation, the oil will be subjected to oil refining methods which include processing steps such as distillation, bleaching, deodorization and filtration which would remove any potential enzyme residue and carrier material if present. It is important to note that during refining, before deodorization, esterified lipid (Cocoa butter substitute/ equivalent) is filtered with a 10um polyethylene (PE) filter and further prior to loading in trucks, Cocoa butter substitute/ equivalent is again filtered using a 1µm PE filter to make sure that no particles (e.g. immobilized enzyme, unbound resin) are present in the oil.

### **3 §170.235 PART 3 OF A GRAS NOTICE: DIETARY EXPOSURE.**

#### **3.1 ESTIMATES OF HUMAN CONSUMPTION AND SAFETY MARGINS**

##### **3.1.1 ESTIMATES OF HUMAN CONSUMPTION**

The lipase covered in this dossier is typically used in modification / esterification of lipids.

The most appropriate way to estimate the human consumption in the case of food enzymes, such as lipase, is by taking into consideration the average maximum human intake of oils and fats in populations across the world, and determining the amount of the lipase which may be present in final foods based on the amount used for any given application.

In this case, the estimation of the consumption of lipase by humans is done by assuming that the entire immobilized enzyme was removed from the inert carrier during the process and all of it showed up in the product and this product was consumed by the population.

*Rhizopus* lipase is immobilized on an inert carrier. The weight percent of *Rhizopus* lipase immobilized on the carrier is between 5-10%. Typically, 0.3-1kg of immobilized *Rhizopus* lipase granules is used per ton of oil/fat. Assuming that the entire enzyme was removed from the inert carrier during the process and all of it showed up in the product, then the maximum amount of enzyme that will be present in final food will not be more than 100ppm<sup>5</sup>. It is very important to note that 100ppm is a very exaggerative estimate as:

- A. Standard refining techniques used will reduce/deactivate any *Rhizopus* lipase present in the interesterified oil
- B. Once the substrate is depleted, enzymatic reaction will not take place
- C. The enzyme tends to degrade under unfavourable conditions of temperature and pH and it does not perform any technological function in the final food.

Hence, if at all the enzyme is present in the final food; it will not exceed 100ppm.

##### **Human Milk Substitutes:**

The intended use for *Rhizopus* lipase covered by this application is for the interesterification of oils for use in human milk substitutes (HMS). The fat content of HMS is usually about 3.5 - 4.5 g/100ml<sup>6,7</sup>. The average daily HMS consumption by healthy infants born at term is 750 ml for the first six month<sup>8,9</sup>.

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<sup>6</sup> Martin C, Ling P, Blackburn G. Review of infant feeding: key features of breast milk and infant formula. Nutrient s. 2016;8(5):279. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4882692/>

<sup>7</sup> First Steps Nutrition Trust (2016) Infant Milk Composition [http://www.firststepsnutrition.org/pdfs/Infant\\_milk\\_composition\\_February2016.pdf](http://www.firststepsnutrition.org/pdfs/Infant_milk_composition_February2016.pdf)

<sup>8</sup> Recommended Dietary Allowances: 10th Edition (1989) National Academies Press <https://www.nap.edu/read/1349/chapter/2#3>

<sup>9</sup> Betapol structured Triglycerides) for use in term and preterm infant formula. (GRN131) <http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm261678.pdf>

Now, assuming the higher fat content of 4.5 g/100ml and a daily intake of 750 mL, then the **maximum daily intake is = 4.5g /100ml x 750ml = 33.75g of fat/day**. According to GRAS notifications 131 and 192 (GRN 131 and GRN 192), FDA notes that it estimated intake of high 2-palmitic vegetable oil in infants as 22 g/day at the mean and 35 g/day at the 90th percentile.

TOS for *Rhizopus* lipase is estimated as 84.66%.

Dose of concentrate is therefore equal to 84.66mg/kg of the raw material (considering the maximum dose to be 100ppm).

It is clear that 84.66 mg is required for 1 kg of the raw material.

Therefore, for 33.75g of raw material  $(84.66/1000 \times 33.75)=2.857\text{mgTOS/day}$ .

For an average infant weighing 2.97 kg, the maximum daily intake will=  $2.857/2.97=0.962\text{mgTOS/kg body weight/day}$ .

### Safety Margin

The sub-chronic 90 days oral toxicity study showed a No Observed Adverse Effect Level (NOAEL) of at least 846.6 mg TOS/kg/day. [Refer [Section 6.4](#) for details]

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL by the estimated human consumption value. As was shown above, the estimated human consumption value of the food enzyme is **0.962mgTOS/kg body weight/day**. Consequently, the MoS is **880**.

**Margin of Safety (MoS) = 846.6/0.962 = 880**

As explained above, the estimated human consumption value of the food enzyme is highly exaggerated. Moreover, the NOAEL was based on the highest dose administered, and is therefore to be considered as a minimum value. Therefore, the actual MoS in practice will be some magnitudes higher. Consequently, there are no safety reasons for laying down maximum levels of use.

### Cocoa Butter Substitutes/ Equivalents

The intended use for *Rhizopus* lipase covered by this application is for the interesterification of oils for use Cocoa Butter Substitutes / Equivalents (CBEs).

According to the data available at **National Health and Nutrition Examination Survey (NHANES)** 2011-2012, Table 1 provides a summary of the Estimated daily intake of Cocoa Butter Equivalent from Authorized Food-Uses in the U.S. by Population Group and Table 2 provides Summary of the Estimated daily per kilogram body weight intake of Cocoa Butter Equivalent from Authorized Food-Uses in the U.S. by Population Group.

Table 3.1.1- 1: Summary of the Estimated Daily Intake of Cocoa Butter Equivalent from Authorized Food-Uses in the U.S. by Population Group (2011- 2012 NHANES Data)

Population Group	Age Group (Years)	All person consumption (Gram/day)		All User consumption (grams/day)			
		Mean	90 <sup>th</sup> percentile	% Users	n	Mean	90 <sup>th</sup> percentile
Infants	Upto 1	<0.1*	NA	1.2	7	1.8	3.7
Young Children	1-3	0.9	2.7	30.2	150	2.9	7.3
Children	4-11	1.7	5.7	43	509	4	8.5
Female Teenagers	12-19	1.7	5.0	33.9	169	5	12.1
Male Teenagers	12-19	1.6	5.7	26.3	140	6	12.6
Female Adults	20 and up	1.8	5.8	36.8	743	4.9	9.7
Male Adults	20 and up	1.9	6.2	35.2	651	5.5	12.7
Total Population	All ages	1.8	5.8	35.4	2,369	5.0	11.2

NA: Not available \*: Value was not considered when assessing the relative contribution of specific food uses to total cocoa butter substitute consumption because mean and 90th percentile intake estimates based on sample sizes of less than 30 and 80, respectively, may not be considered statistically reliable due to the limited sampling size (LSRO 1995)

The maximum daily consumption of cocoa butter substitutes is by male adults and has a value of 12.7 grams/kg at 90<sup>th</sup> percentile.

TOS for *Rhizopus* lipase is estimated as 84.66%.

Dose of concentrate is therefore equal to 84.66mg/kg of the raw material (considering the maximum dose to be 100ppm).

It is clear that 84.66 mg is required for 1 kg of the raw material.

Therefore, for 12.7 g of raw material  $(84.66/1000 \times 12.7)=1.07\text{mgTOS/day}$ .

Considering the average weight of male adult to be 60 kg =  $1.07/60 = 0.017 \text{ mgTOS/kg body weight/day}$



### Safety Margin

The sub-chronic 90 days oral toxicity study showed a No Observed Adverse Effect Level (NOAEL) of at least 846.6 mg TOS/kg/day. [Refer [Section 6.4](#) for details]

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL by the estimated human consumption value. As was shown above, the estimated human consumption value of the food enzyme is **0.017 mgTOS/kg body weight/day**.

Consequently, the MoS is 49,800

$$\text{Margin of Safety (MoS)} = 846.6/0.017 = 49,800$$

For all the above calculations, it must be emphasized that the estimated human consumption is based on conservative assumptions and represents a highly exaggerated value because of (among others) the following reasons:

- It is assumed that all producers of the above mentioned foodstuffs use the *Rhizopus* lipase from *Aspergillus niger* group spp., and apply the highest recommended level.
- It is assumed that the entire enzyme dosage is carried over to the final product(s), although this would not be the case in reality. Infact, the enzyme does not perform any technological function in the final food.
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed daily over the course of a lifetime.
- It is assumed that the average weight of an infant is 2.97 kg and that of an adult is 60 kg.
- The NOAEL was based on the highest dose administered, and is therefore to be considered as a minimum value. Therefore, the actual MoS in practice will be some magnitudes higher. Consequently, there are no safety reasons for laying down maximum levels of use.

## **4 §170.240 PART 4 OF A GRAS NOTICE: SELF-LIMITING LEVELS OF USE**

### **4.1 SELF-LIMITING LEVELS OF USE**

There are no self-limiting levels of use of *Rhizopus* lipase from *Aspergillus niger* agg. (strain FL100SC) in esterification/ modification of oils and fats as described in [Section 3](#) of the dossier.

Immobilized *Rhizopus* lipase from *Aspergillus niger* agg. (strain FL100SC) is insoluble and physically stable in oil; furthermore, after coming in contact with the enzyme preparation, the oil will be subjected to oil refining methods which include processing steps such as filtration and deodorization/distillation which would inactivate and remove any potential enzyme residues from final food. The presence of the enzyme lipase in final food is inconsequential, as the substrate is depleted / absent or unfavourable conditions of pH and temperature exist owing to which the enzyme does not elicit any technological effect on the final food. Also, the enzyme tends to degrade under unfavourable conditions of temperature and pH and it does not perform any technological function in the final food.

Lipase covered in this dossier is used a processing aid and does not perform any technological function in the final food. The immobilized enzyme is recovered once the enzymatic reaction is completed and it is unlikely to be present in the final food. In the worst case scenario, if enzyme is leached into the final food, its presence in final food (if at all it is present), will not affect the quality of food in anyway.

**5     §170.245 PART 5 OF A GRAS NOTICE: EXPERIENCE BASED ON  
COMMON USE IN FOOD BEFORE 1958**

The statutory basis for our conclusion of GRAS status in the notice is not based on common use in food. Our notice does not include evidence of a substantial history of consumption of the notified substance for food use by a significant number of consumers prior to January, 1958.

## **6 § 170.225 PART 6, GRAS NOTICE: NARRATIVE**

### **6.1 SAFETY OF DONOR AND PRODUCTION STRAIN**

The source material (production strain) that is used for the production of lipase covered in this dossier is *Aspergillus niger* agg. (strain FL100SC). The organism is deposited with the American Type Culture Collection (ATCC), and the safe deposit number is SD-6846. The organism is derived from the parental strain *Aspergillus niger* (strain ASNSC).

The parental strain *ASNSC*, and production strain *FL100SC* were tested by CBS (Centraalbureau voor Schimmelcultures) for mycotoxin production, and were shown not to produce any mycotoxins, establishing the safe nature of the parental and the production strains. This was further substantiated by the lack of toxicity of the enzyme when tested in various *in vitro* and *in vivo* toxicity model systems. The strains have also been analyzed for its potential to produce ochratoxins using techniques such as PCR-RFLP methods and is considered as non ochratoxigenic fungus. (Saroj et al, 2016)

### **6.2 SAFETY OF ENZYME**

*Rhizopus* lipase has a long history of use in food processing. *Rhizopus* lipase is approved by Health Canada, Brazil Food Authority, Légifrance, GB list of China and Food standards of Australia and New Zealand. Also, FDA, had no questions regarding the conclusion that *Rhizopus* lipase enzyme preparation is GRAS under the intended conditions of use (GRN 000216)

In the early 1960s the FDA issued opinion letters recognizing, inter alia, that enzymes from *Aspergillus niger* can be ‘generally regarded as safe’ (GRAS) under the condition that non-pathogenic and non-toxigenic strains and current good manufacturing practices be used in production (Schuster et al 2002). In the Food Standards Code-1.3.3-processing aids of FSANZ, lipase from *Aspergillus niger* is listed as safe for use in food.

Additionally, the *Rhizopus* lipase covered in this dossier and produced by *Aspergillus niger* agg. (strain FL100SC), does not contain mycotoxins or heavy metals above specified detection levels. (Refer [Annex E1](#) & [Annex D](#)). Further, it does not contain any detectable amount of full length recombinant DNA as determined by the absence of amplicon for full length recombinant gene of lipase. This confirmed the absence of recombinant DNA in the product.

The safety of lipase was further substantiated by lack of toxicity, when the food enzyme object was tested in standard in vitro (OECD 471 - Ames’ test and OECD 473 - chromosomal aberrations studies) and in vivo (OECD 408 – Repeated dose 90-day oral toxicity studies) model systems.

### 6.2.1 **ALLERGENICITY**

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.

It is pertinent to note that majority of proteins are not food allergens, and owing to their safe nature, it is understandable that enzyme proteins used in food are not homologous to known food allergens. It is also clear that very small quantities of the food enzyme(s) are used during food processing, resulting in miniscule quantities of the food enzyme(s) in the final food. Goodman et al (2008) discuss that a high concentration generally equals a higher risk of sensitization, whereas a low level in the final food equals a lower risk. Additionally, it must be noted that the food enzyme protein undergoes denaturation under various conditions of food processing, resulting in loss of its tertiary structure. In general, these alterations in conformation are associated with decrease in the antigenic reactivity in humans. Usually, denatured proteins are much less immunogenic than the corresponding native proteins [Takai *et al.*, 1997; Takai *et al.*, 2000 (as cited in Koyanagi et al. 2010); Valenta, 2002; Kikuchi *et al.*, 2006]. Further, residual enzyme(s) still present in the final food will be subjected to digestion in the gastro-intestinal system, which reduces further the risk of enzyme allergenicity. It is believed that small protein fragments resulting from digestion are less likely to be allergenic (FAO/WHO, 2001; Goodman *et al.*, 2008).

At present, there is no single definitive test that can be relied upon to predict allergic responses in humans to newly expressed protein.

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 (Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology).

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

Bioinformatics searches show that *Rhizopus* lipase from *Aspergillus niger* agg. (strain FL100SC) 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. (Refer [Annex I](#)).

A general consensus has been reached that proteins susceptible to gastrointestinal digestion are inherently safer than those that are stable with respect to allergenicity (Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants - CAC/GL 45-2003). With the above understanding as the basis, pepsin digestion test was carried on the test enzyme proteins. It shows that *Rhizopus* 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. (Refer [Annex I](#))

Overall, it is worthwhile noting that:

- a. food enzymes have a long history of safe use in food, with no indication of adverse effects or reactions,
- b. a wide variety of enzyme classes are naturally present in food,
- c. 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,
- d. 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, (bioinformatics, pepsin digestion and scientific literature) ingestion of a food enzyme lipase is not a concern in terms of food allergy.

#### **6.2.2 LEADING ENZYME PUBLICATIONS ON THE SAFETY OF THE ENZYME OR ENZYMES THAT ARE CLOSELY RELATED / SAFETY OF USE IN FOOD**

*Rhizopus oryzae* lipase produced by a fermentation process was subjected to a series of toxicological tests to document the safety for use as a food additive. The enzyme product was examined for acute, subacute and subchronic oral toxicity, and mutagenic potential. An extensive literature search on the production organism has also been conducted. No evidence of (sub)acute oral toxicity or mutagenic potential was found. Administration of the lipase at dosages of 50, 200 and 1000 mg/kg body weight/day for 90 days did not induce noticeable signs of toxicity (Coenen et al, 1997).

FDA, had no questions regarding the conclusion that *Rhizopus lipase* enzyme preparation is GRAS under the intended conditions of use (GRN 000216).



During recent years, genetic engineering techniques have been used to improve the industrial production strains of *Aspergillus niger* and considerable experience on the safe use of recombinant *Aspergillus niger* strains at industrial scale has accumulated. FAO/WHO experts have repeatedly reviewed and accepted enzyme preparations from *Aspergillus niger*, including the organism itself (FAO/WHO 1972, 1978, 1981, 1987, 1990), listing them with an Acceptable Daily Intake of ‘not specified’. The FDA in the United States has accepted numerous enzymes for food use: in the early 1960s the FDA issued opinion letters recognizing, *inter alia*, that lipase from *Aspergillus niger* can be ‘generally regarded as safe’ (GRAS) under the condition that non-pathogenic and non-toxicogenic strains and current good manufacturing practices be used in production (GRN000089, GRN 000111, GRN 000132).

**INFORMATION ON EXISTING AUTHORIZATIONS AND EVALUATIONS**

Table 6.2.2-1 Non-exhaustive list of authorizations of *Rhizopus lipase* and lipase obtained from *Aspergillus niger*

Authority	Description	Reference
Australia/ New Zealand	Lipase from <i>Rhizopus oryzae</i>	<a href="#">Standard 1.3.3 processing aids</a>
	Lipase from <i>Aspergillus niger</i>	
Canada <sup>10</sup>	Lipase from <i>Rhizopus oryzae</i> var.	<a href="#">B.16.100, Table V</a>
	Lipase from <i>Aspergillus niger</i> (pCaHj600/MBin118#11)	
	Lipase from <i>Aspergillus niger</i> var.	
France	Lipase de <i>Rhizopus oryzae</i> FLP-1	<a href="#">Arrêté du 19 Octobre 2006</a>
	Lipase de <i>Rhizopus oryzae</i> .	
	Lipase produite par la souche d' <i>Aspergillus niger</i> modifiée génétiquement LFS-54 contenant un gène codant la lipase de <i>Fusarium culmorum</i> .	
USA <sup>11</sup>	Lipase from <i>Rhizopus oryzae</i>	<a href="#">GRN216</a>
	Lipase from <i>Aspergillus niger</i>	<a href="#">GRN111</a>
	Lipase enzyme preparation derived from a genetically modified strain of <i>Aspergillus niger</i>	<a href="#">GRN296</a>
Brazil	Lipase from <i>Rhizopus oryzae</i>	<a href="#">diario das leis</a>

<sup>10</sup> *Aspergillus niger* var. includes various subspecies of *Aspergillus niger* group. The listing as *Aspergillus niger* var., rather than as simply *Aspergillus niger*, takes into consideration the ambiguity in *Aspergillus niger* group nomenclature.

<sup>11</sup>GRAS affirmations and GRAS notifications

	Lipase from <i>Aspergillus niger</i>	
China	Lipase from <i>Rhizopus oryzae</i>	<a href="#">National Standard of the People's Republic of China: Usage standards of food additives</a>
	Lipase from <i>Aspergillus niger</i>	

### 6.2.3 SAFETY OF USE IN FOOD

In addition to the allergenicity assessment described above, the safety of this lipase has also been established using the Pariza and Johnson (2001) decision tree:

1. Is the production strain genetically modified?

YES

If yes, go to 2

2. Is the production strain modified using rDNA techniques?

YES

If yes, go to 3a

3a. Does the expressed enzyme product which is encoded by the introduced DNA have history of safe use in food?

**YES**, the lipase under study is not new for food processing. Its protein sequence is not similar to known sequences of food allergens. In addition, the enzyme will be inactivated in the food manufacture process.

If yes, go to 3c

3c. Is the test article free of transferable antibiotic resistance gene DNA?

**YES**. No functional antibiotic resistance genes were left in the strain as a result of the genetic modifications. The absence of these genes was verified.

If yes go to 3e

3e. Is all other introduced DNA well characterised and free of attributes that would render it unsafe for constructing microorganisms to be used to produce foodgrade products?

**YES** The genetic modifications are well characterized and specific and the incorporated DNA does not encode and express any known harmful or toxic substances.

If yes, go to 4.

4. Is the introduced DNA randomly integrated to the chromosome?

**YES**, the introduced DNA randomly integrated to the chromosome

If yes go to 5

5. If the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed?

**YES**

If yes go to 6

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?

**YES**

The production strain *Aspergillus niger* agg FL100SC is derived from the parent strain *Aspergillus niger* strain ASNSC. The strain ASNSC is in use for production of pectolytic enzymes for over 15 years at Advanced Enzyme Technologies Ltd.

The parent strain ASNSC is analyzed by two leading mycology centres, i.e. Centraalbureau voor Schimmelcultures (CBS, The Netherlands) and Center for Agriculture and Biosciences International (CABI, UK).

At CBS, the parental *Aspergillus niger* (strain ASNSC) was identified as *Aspergillus* Section *Nigri* species. Phenotypic analysis of *Aspergillus* section *Nigri* strain showed typical structures for an isolate that belongs to the *Aspergillus* section *Nigri*. Analysis of the sequence data (part of the  $\beta$ -tubulin gene and part of the Calmodulin gene amplified and sequenced from gDNA) showed that the ANSC03131 strain shared sequences with isolates of *Aspergillus acidus*, Kozak (redesignated as *Aspergillus luchuensis* by Hong et al, 2013).

At CABI, the multiple unlinked genes Internal Transcribed Spacer (ITS) and Calmodulin were amplified and sequenced from gDNA of parental strain ASNSC. The concatenated genes showed closeness to *Aspergillus tubingensis* and *Aspergillus acidus*. Based on these observations, CABI concluded the parental strain identified, vide CABI reference number Y12/13/H64 IMI 502637, be designated as *Aspergillus niger* agg.

The parent strain ASNSC was thoroughly investigated at Centraalbureau voor Schimmellecultures (CBS, The Netherlands) for its' ability to produce mycotoxin. The results showed that the strain does not produce the studied mycotoxins, which includes mycotoxins produced by *Aspergillus niger*, establishing the safe nature of the mycotoxin. Here, it is worthwhile noting that Hong et al, 2013, reported that extrolite analysis of strains of *Aspergillus luchuensis* showed that they do not produce mycotoxins and therefore can be considered safe for food and beverage fermentations. Further, the strain has also been analyzed for its potential to produce ochratoxins using techniques such as PCR-RFLP methods and is considered as non ochratoxigenic fungus. (Saroj et al, 2016). The studies carried out on the production strain and also other strains of the same species clearly indicate safe nature of the production microorganism.

The parent strain ASNSC is used to produce pectinase. This strain has also been genetically modified to produce enzymes such as pectin lyase, pectin methyl esterase, and polygalacturonase. The enzymes produced by these GMMs have also been evaluated for repeated dose 90 days oral toxicity study (OECD 408) AMES test (OECD 471) and 'In Vitro' mammalian chromosomal aberration test (OECD 473). In all the cases the enzymes produced by the GMMs have been found safe at the highest dosage tested.

The toxicity data available for these various enzymes suggests that there is no safety concern associated with the use of the enzymes produced from ASNSC strains. (Refer [Annex L](#) for the toxicity reports of the mentioned enzymes).

If yes, article is accepted

**Conclusion: Article is accepted.**

### **6.3 SAFETY OF MANUFACTURING PROCESS**

The lipase described in this dossier is manufactured in accordance with current Good Manufacturing Practice for Food (cGMP) and the principles of Hazard Analysis of Critical Control Points (HACCP). Refer [Annex F](#) for details.

## 6.4 SAFETY STUDIES

The safety of the lipase is supported by a standard package of toxicological tests, viz:

- (i) Ames' reverse mutation test (OECD 471),
- (ii) *In vitro* chromosomal aberration test in human lymphocytes (OECD 473)
- (iii) Repeated 90-days oral toxicity (OECD 408).

Sample	: <i>Rhizopus oryzae</i> Lipase from <i>Aspergillus niger</i> agg. (strain FL100SC): Batch no- 011423
Activity	: 5,48,964 FIP U/g
TOS	: 84.91% (Mean of 3 batches, comprising 2 Commercial batches and 1 Tox batch is 84.66% used for dietary exposure calculations in order to account for batch variation)
NOAEL Dose	: 1000 mg/kg of body weight. i.e. 846.6 mg TOS/ kg body weight/day of lipase from <i>Aspergillus niger</i> agg. (strain FL100SC)

The composition and specifications of the test materials (spray-dried unformulated concentrate) are given in the Table below:

Table 6.4-1 Toxicity batch specifications and other details

Batch No.	<b>011423</b>
Ash (%)	8.14
Water (%)	6.95
TOS (%)*	84.91
Activity (FIP U/g)	548,964
LU/mg TOS	646.52
Lead (mg/kg)	Not more than 5 ppm
Salmonella sp. (per 25 g)	Absent by test
Total coliforms (per g)	Not more than 30
Escherichia coli (per 25 g)	Absent by test
Antimicrobial activity	Absent by test
Mycotoxins	Absent by test

\* calculated

#### 6.4.1 **AMES' TEST**

The aim of this in-vitro study was to detect the possible presence of compounds or contaminants in the test substance, lipase from *Aspergillus niger* (strain FL100SC) (Batch no. 011423). This test enables the detection of potential mutagens which act by causing base-pair substitutions or frameshift mutations. Tester strains of *Salmonella typhimurium* are used in this study.

The study was performed according to: OECD. Guideline No. 471, adopted on July 21, 1997. The study was conducted in accordance with the principles of Good Laboratory Practice as published by the OECD in 1998, No.1, ENV/MC/CHEM (98)17.

In order to determine the potential of the *Rhizopus* lipase from *Aspergillus niger* (strain FL100SC) (Batch no. 011423) for its ability to induce gene mutations, the bacterial reverse mutation test was conducted using *Salmonella typhimurium* tester strains viz., TA97a, TA98, TA100, TA102, TA97a, TA98 and TA1535 (Refer [Annex K](#) for details on Methods and Results)

Based on the results it is concluded that lipase tested at and upto 5000.00 µg /plate concentration did not induce any mutations in the presence and absence of microsomal enzymes (S-9 fraction) and is therefore non-mutagenic in this Bacterial Reverse Mutation Test.

#### 6.4.2 **CHROMOSOMAL ABERRATION TEST**

The aim of this in vitro study was to detect the possible presence of compounds or contaminants in the test substance, *Rhizopus* lipase from *Aspergillus niger* (strain FL100SC) (batch no. 011423), capable to induce chromosomal breakage (clastogenesis) in cultured human lymphocytes.

This test enables the detection of any chromosomal and chromatid structural aberration in the cells blocked at the metaphase stage.

The study was performed according to: OECD. Guideline No. 473, adopted on September 26, 2014. The study was conducted in accordance with the principles of Good Laboratory Practice as published by the OECD in 1998, No.1, ENV/MC/CHEM(98)17.

The study was conducted to evaluate the clastogenic potential of *Rhizopus* lipase from *Aspergillus niger* (strain FL100SC) (Batch no. 011423), as judged by the ability to induce chromosomal aberrations in an *in vitro* human lymphocyte system (Refer [Annex K](#) for details on Methods and Results)

Based on the results obtained it is concluded that *Rhizopus* lipase from *Aspergillus niger* (strain FL100SC) tested at and upto 5 mg/ml of culture concentration did not induce chromosome aberrations in human lymphocyte cells *in vitro* and therefore is non-clastogenic in the presence and absence of microsomal enzymes (S-9 fraction).



### 6.4.3 ASSESSMENT OF SYSTEMIC TOXICITY

The aim of this study was to investigate the clinical, haematological and histological toxicity potential of *Rhizopus* lipase from *Aspergillus niger* (strain FL100SC) (Batch No. 011423), following a 90-days oral administration in male and female rats receiving a daily treatment.

The study was performed according to: OECD. Guideline No. 408, adopted on September 21, 1998. The study was conducted in accordance with the principles of Good Laboratory Practice as published by the OECD in 1998, No.1, ENV/MC/CHEM(98)17.

The repeated dose 90 day oral Toxicity study followed by 4 week recovery period was designed and conducted to determine the toxicity profile of lipase from *Aspergillus niger* (strain FL100SC) when administered daily for 90 days in the Sprague Dawley rats. (Refer [Annex K](#) for details on Method and Observation/Results)

The repeated dose 90 day oral Toxicity study followed by 4 week recovery period was designed and conducted to determine the toxicity profile of lipase from *Aspergillus niger* (strain FL100SC). The lipase was administered to the Sprague Dawley rats at the dose level of 250 mg/kg, 500mg/kg and 1000 mg/kg body weight for 90 days. The rats were examined daily for sign of toxicity, morbidity and mortality OR for assessment of toxicity. (Refer [Annex K](#) for details on Method and Observation/Results)

Based on the findings of study, the No observed Adverse Effect level (NOAEL) of *Rhizopus* lipase from *Aspergillus niger* (strain FL100SC) in Sprague Dawley rat via oral route, over a period of 90 days was found to be 1000 mg/kg body weight in male and female animals. This corresponding to 548,964 FIP U/kg/day for male & female rats, which in turn equates to 846.6 mg TOS/kg/day.

Summarizing the results obtained from the several toxicity studies the following conclusions can be drawn:

- No mutagenic or clastogenic activity were observed under the given test conditions
- The sub-chronic oral toxicity study showed a No Observed Adverse Effect Level (NOAEL) of at least 846.6 mg TOS/kg body weight/day.

### 6.5 SAFETY MARGIN

The safety margin is calculated as dose level with no adverse effect (NOAEL) divided by the estimated human consumption.

Table 6.5-1 Safety of margin of *Rhizopus* lipase in different applications

Application	NOAEL of <i>Rhizopus</i> lipase in mg TOS/kg body weight/day.	Theoretical Maximum Daily Intake (mg TOS/kg body weight/day).	Margin of safety
Human milk substitutes	846.6	0.962	880

Cocoa butter equivalents	846.6	0.017	49,800
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As is explained in Part 3 of the dossier, the estimated human consumption value of the food enzyme is highly exaggerated. Moreover, the NOAEL was based on the highest dose administered, and is therefore to be considered as a minimum value. Therefore, the actual Margin of safety (MoS) in practice will be some magnitudes higher. Consequently, there are no safety reasons for laying down maximum levels of use.

## 6.6 EXPERT PANEL REPORT

An independent panel of recognized experts qualified by their scientific and/or medical training and international experience to evaluate the safety of food and food ingredients was requested by Advanced Enzymes Technologies Ltd to determine the GRAS status of *Rhizopus* lipase expressed in *Aspergillus niger* agg. (strain FL100SC), for use as a processing aid in the modification and/or esterification of lipids, including, but not limited to human milk substitutes.

The panel consisted of Dr. Micheal Pariza<sup>12</sup>, Joseph F. Borzelleca<sup>13</sup> and Dr. Dennis Bier<sup>14</sup>. They concluded that “Advanced Enzyme's *Aspergillus niger* agg. (strain FL100SC) is safe and appropriate to use for the manufacture of food-grade *Rhizopus oryzae* (*Rhizopus*) lipase enzyme. We further conclude that the *Rhizopus* lipase enzyme preparation produced by *A. niger* agg. (strain FL100SC), manufactured in a manner that is consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications, is GRAS (Generally Recognized As Safe) for use as a processing aid in the modification/esterification of lipids including, but not limited to, human milk fat substitute and cocoa butter substitute, where the lipase enzyme is either not present in the final food/feed or present at trace levels as inactive protein having no functional or technical effect.” (Refer [Annex M](#) for the complete expert panel report)

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<sup>12</sup> Dr. Micheal Pariza, Ph.D, Professor Emeritus, University of Wisconsin-Madison

<sup>13</sup> Joseph F. Borzelleca, Professor Emeritus, Virginia Commonwealth University School of Medicine

<sup>14</sup> Dr. Dennis Bier, Professor, Pediatrics-Nutrition, Children's Nutrition Research Center, Baylor College of Medicine

### **General Recognition of the Safety of *Rhizopus* lipase from *Aspergillus niger***

As noted in the sections above, parental organism, production organism as well as the enzyme preparations derived therefrom, are well recognized by qualified experts as being safe. Published literature, government laws and regulations, reviews by experts, as well as Advanced Enzymes' own unpublished safety studies, support such a conclusion.

*Aspergillus niger* and *Rhizopus oryzae* are widely used by enzyme manufacturers around the world for the production of enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. *Aspergillus niger* is a considered safe host for enzyme production as well as it is considered non ochratoxigenic fungus.

The lipase covered in this dossier does not contain detectable amount of full length recombinant DNA. Absence of transferable rDNA sequences in the enzyme preparation has also been established. It does not contain mycotoxins or heavy metals above specified detection levels.

Immobilized *Rhizopus* lipase from *Aspergillus niger* (strain FL100SC) is insoluble in oil; furthermore, after coming in contact with the enzyme preparation, the oil will be subjected to oil refining methods which include processing steps such as filtration and deodorization/distillation which would remove any potential enzyme residues from final food. The presence of the enzyme lipase in final food is inconsequential, as the substrate is depleted / absent or unfavourable conditions of pH and temperature exist owing to which the enzyme does not elicit any technological effect on the final food.

The lipase described in this dossier is manufactured in accordance with current Good Manufacturing Practice for Food (cGMP) and the principles of Hazard Analysis of Critical Control Points (HACCP).

It has also been established that the immobilized enzyme does not get leached out into the food. Also, immobilization support/ carrier, inter alia, do not transfer its constituents to foodstuffs in quantities which could endanger human health or bring about an unacceptable change in the composition of the foodstuff or deterioration in the organoleptic characteristics thereof.

Using the decision tree of Pariza and Johnson (2001) the *Rhizopus* lipase from *Aspergillus niger* (strain FL100SC) enzyme preparation was determined to be acceptable for the proposed uses. For verification whether the NOAEL is sufficient to support a 100- fold safety margin in

the intended uses, the safety studies conducted on the lipase produced by the production strain described in this submission established a NOAEL at 846.6 mg TOS/kg/day. Based on a worst-case scenario, the maximum daily intake for infants in case of human milk substitutes is 0.962mgTOS/kg body weight/day. The maximum daily intake in case of cocoa butter equivalents is 0.017 mgTOS/kg body weight/day (The maximum daily consumption of cocoa butter substitutes is by male adults and has a value of 12.7 grams/kg at 90<sup>th</sup> percentile).

Therefore, the use of lipase in preparation of human milk substitutes and cocoa butter equivalents is not expected to result in adverse effects to humans. A safety margin of 880 (for human milk substitutes) and 49,800 (for cocoa butter equivalents) exists between the established NOAEL and the estimated worst case maximum daily human cumulative exposure level.

Based on the available data from the literature and generated by Advanced Enzyme Technologies Ltd., the company has concluded that *Rhizopus* lipase from *Aspergillus niger* (strain FL100SC) is safe and suitable for modification/ esterification of lipids.

## 7 §170.255 PART 7 OF A GRAS NOTICE: LIST OF SUPPORTING DATA AND INFORMATION IN YOUR GRAS NOTICE.

### 7.1 ANNEXES

<b>Annex</b>	<b>Description</b>	<b>Reference</b>	<b>Pages</b>
<a href="#">Annex A1</a>	Method of Analyses	-	1-17
<a href="#">Annex A2</a>	Analytical method of lipase by FIP		1-8
<a href="#">Annex B</a>	Technical Report on the characterization of <i>Rhizopus oryzae</i> lipase (EC 3.1.1.3) produced by genetically modified <i>Aspergillus niger</i> (strain FL100SC)	ROL/AET/P RO/014A	1-23
<a href="#">Annex C</a>	Technical Report on the characterization of <i>Rhizopus oryzae</i> lipase (EC 3.1.1.3) produced by genetically modified <i>Aspergillus niger</i> (strain FL100SC)	ROL/AET/P RO/014B	1-30
<a href="#">Annex D</a>	Heavy Metals Analysis – TUV Nord Group	0081300765, 0081300766, 0071400444	1-3
<a href="#">Annex E1</a>	Mycotoxin Analysis – TNO Triskelion BV	20121-0788 20121-0789 20121-0790	1-3
<a href="#">Annex E2</a>	Evaluation of antimicrobial activity of lipase enzyme produced by <i>Aspergillus niger</i> agg (strain FL100SC), and antimicrobial production potential of the production strain.	AET/POL/ Micro/008A	1-23
<a href="#">Annex F</a>	Hazard Analysis and Critical Control Points (HACCP) of <i>Rhizopus oryzae</i> lipase (EC 3.1.1.3) produced by genetically modified <i>Aspergillus niger</i> (strain FL100SC)	-	1-12
<a href="#">Annex G</a>	Safety considerations for biotechnology- GILSP by OECD (1992)	-	1-45
<a href="#">Annex H1</a>	Identification of production strain	Det 13.094	1-11
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<a href="#">Annex I</a>	Allergenicity Assessment – <i>Rhizopus oryzae</i> lipase produced by <i>Aspergillus niger</i> agg (strain FL100SC)	-	1-8
<a href="#">Annex J</a>	Report on assessment of the leakage of active enzyme and /or immobilization support materials/resins into food.	ROL/AET/F L/002	1-14
<a href="#">Annex K</a>	Combined file of toxicity studies: Bacterial reverse mutation test of <i>Rhizopus oryzae</i> lipase	IIT-17782	1-52
	<i>In vitro</i> mammalian chromosome aberration test of <i>Rhizopus oryzae</i> lipase in human lymphocytes	IIT-17783	1-61
	Repeated dose 90-day oral Toxicity study with <i>Rhizopus oryzae</i> lipase by daily gavage in the rat followed by a 4 week recovery period	IIT-17956	1-531
<a href="#">Annex L</a>	Toxicity reports of Pectin lysase, Pectin(methyl) esterase, polygalacturonase and pectinase	-	-
<a href="#">Annex M</a>	Expert Panel Report	-	1-3



## 7.2 BIBLIOGRAPHY-REGULATIONS / REPORTS BY IMPORTANT AGENCIES (BBR)

1. Arrêté du 19 Octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la fabrication de certaines denrées alimentaires. ; (2006) | [http://www.legifrance.gouv.fr/affichTexte.do;jsessionid=C8C7772AB2563855B331A28AD00728B6.tpdjo09v\\_1?cidTexte=LEGITEXT000020667468&dateTexte=20130508](http://www.legifrance.gouv.fr/affichTexte.do;jsessionid=C8C7772AB2563855B331A28AD00728B6.tpdjo09v_1?cidTexte=LEGITEXT000020667468&dateTexte=20130508)
2. Australia New Zealand Food Standards Code - Standard 1.3.3 - Processing Aids, clause 15, 16 and 17. ; (2006) | <http://www.comlaw.gov.au/Details/F2012C00064>
3. Brazil Diario Das Leis <https://www.diariodasleis.com.br/tabelas/211121.pdf>
4. Canadian Food and Drug Regulations, C.R.C., c. 870, Section B.16.100, Table V.; (2006) | [http://laws-lois.justice.gc.ca/eng/regulations/C.R.C.%2C\\_c.\\_870/page-160.html#docCont](http://laws-lois.justice.gc.ca/eng/regulations/C.R.C.%2C_c._870/page-160.html#docCont)
5. CDC (2014). National Health and Nutrition Examination Survey (NHANES): 2011-2012. Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: [http://www.cdc.gov/nchs/nhanes/search/nhanes11\\_12.aspx](http://www.cdc.gov/nchs/nhanes/search/nhanes11_12.aspx) [Page last updated: October 31, 2014]
6. Chinese Standards for Food Additives - GB2760-2015 [https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Standard%20for%20Food%20Additive%20Use%20-%20GB2760-2015\\_Beijing\\_China%20-%20Peoples%20Republic%20of\\_4-28-2015.pdf](https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Standard%20for%20Food%20Additive%20Use%20-%20GB2760-2015_Beijing_China%20-%20Peoples%20Republic%20of_4-28-2015.pdf)
7. Dauvrin T, Groot G, Maurer K-H, de Rijke D, Ryssov-Nielsen H, Simonse M and Sorensen TB Working group on consumer allergy risk from enzyme residues in food. An Amfep expert group evaluation study; (1998); Amfep Publication <http://www.amfep.org/sites/default/files/consumer-allergy.pdf>
8. EFSA Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA; (2007); The EFSA Journal; 587; 1-16 | <http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2007.587/epdf>
9. FAO/WHO (2001). Evaluation of allergenicity of genetically modified foods. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology.; (2006) | [http://www.who.int/foodsafety/publications/biotech/en/ec\\_jan2001.pdf](http://www.who.int/foodsafety/publications/biotech/en/ec_jan2001.pdf)
10. FAO/WHO (2006). General Specifications and Considerations for Enzyme Preparations Used in Food Processing. Compendium of food additive specifications, sixty-seventh meeting. FAO JECFA Monographs 3, 2006 (ISBN 92-5-105559-9), 63-67.; (2006) | <ftp://ftp.fao.org/docrep/fao/009/a0675e/a0675e00.pdf>, last visited on 8 May 2013
11. Health Canada and Environment Canada joint publication; Draft screening assessment for *Aspergillus awamori* ATCC 22342 (=A. *niger* ATCC 22342) and *Aspergillus brasiliensis* ATCC 9642; (2014) | <http://www.ec.gc.ca/ese-ees/default.asp?lang=En&n=87F8F8B9-1>
12. JECFA; Inventory of substances used as Processing Aids (IPA) ; (2012); Joint FAO/WHO Food Standards Programme Codex Committee on Food Additives; Forty Fourth Session; 40-56 | [ftp://ftp.fao.org/codex/meetings/CCFA/ccfa44/fa44\\_inf3e.pdf](ftp://ftp.fao.org/codex/meetings/CCFA/ccfa44/fa44_inf3e.pdf)
13. List of Codex Specifications for Food Additives; (2013); CAC/MISC 6-2013 |

- [http://www.codexalimentarius.org/input/download/standards/9/CXA\\_006e.pdf](http://www.codexalimentarius.org/input/download/standards/9/CXA_006e.pdf)
14. OECD Principles on Good Lab Practice(1998)Series on Principles of Good Laboratory Practice and Compliance Monitoring; 1-41  
[http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/MC/CHEM\(98\)17&docLanguage=En](http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/MC/CHEM(98)17&docLanguage=En)
  15. OECD Test No. 471: Bacterial Reverse Mutation Test(1997)OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing; 1-11 <http://www.oecd-ilibrary.org/docserver/download/9747101e.pdf?expires=1406711627&id=id&accname=guest&checksum=A1D7097B5DDBAD66775DC1968812499D>
  16. OECD Test No. 473: *In Vitro* Mammalian Chromosome Aberration test(1997)OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing; 1-10 | <http://www.oecd.org/chemicalsafety/risk-assessment/1948434.pdf>
  17. OECD Test No. 408: Repeated dose 90-day oral toxicity study in rodents(1998)OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing; 1-10 | <http://www.oecd-ilibrary.org/docserver/download/9740801e.pdf?expires=1406712563&id=id&accname=guest&checksum=89F81777E38FA1ED3B9FFAC5CA5A2E49>
  18. 40 US-CFR-Part 725: Reporting requirements and review processes for microorganisms; Sub-Part G: General exemptions for new microorganisms; (2011); United States of America Code of Federal Regulations; 618-623  
<https://www.law.cornell.edu/cfr/text/40/part-725/subpart-G>
  19. US-EPA; *Aspergillus niger* final risk assessment. TSCA Section 5(h)(4) Exemption; (1997); Final Decision Document. United States Environmental Protection Agency; 1-24| [http://www.epa.gov/biotech\\_rule/pubs/fra/fra006.htm](http://www.epa.gov/biotech_rule/pubs/fra/fra006.htm)
  20. USDA (2012b). Agricultural Research Service. What We Eat in America, NHANES 2001-2002, Individuals 2 Years and over (excluding breast-fed children). Nutrient Intakes: Mean Amount Consumed Per Individual, One Day. [www.ars.usda.gov/ba/bhnrc/fsrg](http://www.ars.usda.gov/ba/bhnrc/fsrg)
  21. US-FDA; GRN111: Lipase from *Aspergillus niger*; (2002); Agency Response Letter GRAS Notice No. GRN 000111; 1-2 | <http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm153922.htm>
  22. US-FDA; GRN 296: lipase enzyme preparation derived from a genetically modified strain of *Aspergillus niger*; (2009).  
<http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm189887.htm>
  23. US-FDA GRN 216 Lipase enzyme preparation from *Rhizopus oryzae*. (2006b).  
<http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/UCM269107>.
  24. US-FDA GRN 113 Lipase enzyme preparation from *Aspergillus oryzae*. (2002).  
<http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/UCM260973>.
  25. US-FDA GRN 000089 Protease enzyme from *A. niger* for Meat Tenderizing (2002)  
<http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm266873.pdf>

26. US-FDA GRN 000132 Lactase Enzyme Preparation Produced by *Aspergillus niger* (2003)  
<http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/UCM263136>
27. US-FDA GRN 131 Betapol™ (Structured Triglycerides) (2003).  
<http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm261678.pdf>
28. US-FDA GRN 192 High 2-palmitic vegetable oil. (2006a)  
<http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/UCM269061>.
29. US-FDA GRN 217 Tailored triglycerides containing approximately 12 percent medium-chain fatty acids. (2006c)  
<http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/UCM269108>.
30. US-FDA GRN 81. Lipase from *Candida rugosa*. (2001a).  
<http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/UCM266653>.
31. US- CFR Title 21(b). Part 184- Direct food substances affirmed as generally recognized as safe. Section §184.1420-Lipase enzyme preparation derived from *Rhizopus niveus*.  
<https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=184.1420>
32. US-CFR Title 21(b). Part 184—Direct food substances affirmed as generally recognized as safe. Section §184.1259-Cocoa butter substitute. Available at: [http://www.ecfr.gov/cgi-bin/text-idx?SID=2d47263f39ae98e3eed78f55bf251dbc&mc=true&node=se21.3.184\\_11259&rgn=div8](http://www.ecfr.gov/cgi-bin/text-idx?SID=2d47263f39ae98e3eed78f55bf251dbc&mc=true&node=se21.3.184_11259&rgn=div8)
33. US-FDA GRN 654 Coberine™ N707 (Cocoa Butter Equivalent (eCBE))  
<http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm516029.pdf>

### 7.3 BIBLIOGRAPHY-LITERATURE REFERENCES (BBL)

34. Abarca ML, Accensi F, Cano J and Cabanes FJ; Taxonomy and significance of black aspergilli; (2004); Antonie van Leeuwenhoek; 86; 33-49
35. Alastruey-Izquierdo A, Mellado E, and Cuenca-Estrella M; Current section and species complex concepts in *Aspergillus*: recommendations for routine daily practice; (2012); Annals of New York Academy Sciences; 1273; 18-24
36. Aravindan R, Anbumathi P and Viruthagiri T; Lipase applications in food industry Indian Journal of Biotechnology Vol 6, April 2007, pp 141-158.
37. Bindslev-Jensen C, Stahl Skov P, Roggen EL, Hvass P and Sidelmann Brinch D ; Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry.; (2006); Food and Chemical Toxicology; 44; 1909–1915
38. Bornscheuer UT. Enzymes in lipid modification: From classical biocatalysis with commercial enzymes to advanced protein engineering tools. OCL 2013; 20(1): 45-49.
39. Coenen TM, Aughton P, Verhagen H. Safety evaluation of lipase derived from *Rhizopus oryzae*: summary of toxicological data. Food Chem Toxicol. 1997 Mar-Apr;35(3-4):315-22.

40. De Paula, A.V., Nunes, G.F.M., Silva, J.L. Screening of Food Grade Lipases to be used in Esterification and Interesterification Reactions of Industrial Interest. *Appl Biochem Biotechnol* (2010) 160:1146–1156
41. Douglass JS, Barraij LM, Tennant DR, Long WR and Chaisson CF ; Evaluation of the Budget Method for screening food additive intakes.; (1997); *Food Additives and Contaminants*; 14; 791-802
42. Ferreira-S D Sandoval,G. Plou F, Valero F. The potential use of lipases in the production of fatty acid derivatives for the food and nutraceutical industries *Electronic Journal of Biotechnology* (2013)
43. First Steps Nutrition Trust (2016) Infant Milk Composition
44. Frisvad, J. C., Larsen, T. O., Thrane, U., Meijer, M., Varga, J., Samson, R. A., et al. ; Fumonisin and ochratoxin production in industrial *Aspergillus niger* strains; (2011); *PLoS ONE*; 6; e23496
45. Goodman RE, Vieths S, Sampson HA, Hill D, Ebisawa M, Taylor SL and van Ree R ; Allergenicity assessment of genetically modified crops--what makes sense?; (2008); *Nat Biotechnol*; 26; 73-78
46. Hansen SC ; Acceptable daily intake of food additives and ceiling on levels of use.; (1966); *Food Cosmet Toxicol*; 4; 427–432
47. Hong S-B, Lee M, Kim D-H, Varga J, Frisvad JC, et al.; *Aspergillus luchuensis*, an Industrially Important Black *Aspergillus* in East Asia. (2013) ; *PLoS ONE*; 8(5); 1-9.
48. Houde A., Kademi A, Leblanc D.; Lipases and their industrial applications: *Applied Biochemistry and Biotechnology*; July–September 2004, Volume 118, Issue 1-3, pp 155-170.
49. Kadivar, S., De Clerq, N., de Walle, D. V., and Dewettinck, K. 2014. Optimisation of Enzymatic Synthesis of Cocoa Butter Equivalent from High oleic Sunflower Oil. *J Sci Food Agric* 2014; 94: 1325–1331
50. Kikuchi Y, Takai T, Kuhara T, Ota M, Kato T, Hatanaka H, Ichikawa S, Tokura T, Akiba H, Mitsuishi K, Ikeda S, Okumura K and Ogawa H ; Crucial commitment of proteolytic activity of a purified recombinant major house dust mite allergen Der p 1 to sensitization towards IgE and IgG responses.; (2006); *J Immunol*; 177; 1609-1617
51. Koyanagi S, Murakami T, Nakashima K, Maeda T, Miyatsu Y, et al.; Characterization of the Recombinant Der f 2 Mutant C8/119S and Evaluation of C8/119S in a rDer f 2-Sensitized Rhinitis Mice Model; (2010); *J Aller Ther*; 1; 105 (doi:10.4172/2155-6121.1000105)
52. Lipp, M. and Aravindan, E. (1998a) Review of cocoa butter and alternative fats for use in chocolate Part A. Compositional data. *Food Chem.*, 62, 73–97.
53. Macrae AR (1983) Lipase-Catalyzed Interesterification of Oils and Fats. *JAOCS*, vol. 60, no. 2 (February 1983)/243A
54. Martin C, Ling P, Blackburn G. Review of infant feeding: key features of breast milk and infant formula. *Nutrient s.* 2016; 8(5):279.
55. Pariza MW, Johnson EA; Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. ; 2001; *Regul Toxicol Pharmacol* ; 33; 1-14

56. Perrone G, Stea G, Epifani F, Varga J, Frisvad JC and Samson R; *Aspergillus niger* contains the cryptic phylogenetic species *A. awamori*; (2011); Fungal Biology; 115; 1138-1150
57. Recommended Dietary Allowances: 10th Edition (1989) National Academies Press.
58. Samson RA, Hong S-B and Frisvad JC; Old and new concepts of species differentiation in *Aspergillus*.; (2006); Medical Mycology ; 44; 1; S133-S148
59. Samson RA, Varga J; *Aspergillus* systematics in the genomic era; (2007); Studies in Mycology; 59; Entire Monograph.
60. Schuster E, Dunn-Coleman N, Frisvad J, van Dijck P; On the safety of *Aspergillus niger* – a review; (2002); Applied Microbiology and Biotechnology ; 59; 426-435
61. Saroj D, Dengeti S, Gupta A; Molecular Affirmation of *Aspergillus luchuensis* a Niger Group Species as a Non Ochratoxigenic Fungus (2016) BAOJ Microbio 2016 Volume 2: Issue 2: 009.
62. Sharma. R.; Chisti, Y.; Banerjee, U.C. (2001). Production, purification, characterization, and applications of lipases. Biotechnol Adv, 19: 627-662.
63. Valenta R, Campana R, Marth K, van Hage M; Allergen-specific immunotherapy: from therapeutic vaccines to prophylactic approaches; (2012); J Int Med; 272; 144-157



## Bonnette, Richard

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**Subject:** FW: Re-submission of GRN 708

**From:** Deepti Sood [mailto:deepti@advancedenzymes.com]  
**Sent:** Sunday, May 20, 2018 11:41 PM  
**To:** Bonnette, Richard <Richard.Bonnette@fda.hhs.gov>  
**Cc:** Harshada Chandan <harshada@advancedenzymes.com>; Srinivasan, Jannavi <Jannavi.Srinivasan@fda.hhs.gov>  
**Subject:** Re: Re-submission of GRN 708

Hi Richard,

The information provided to you in Annex K is releasable.

Best Regards,  
Deepti

On Fri, May 18, 2018 at 5:28 PM, Bonnette, Richard <[Richard.Bonnette@fda.hhs.gov](mailto:Richard.Bonnette@fda.hhs.gov)> wrote:

Deepti,

Our filing team has been reviewing the submission over the last few days and we have a quick question regarding a statement of confidentiality in Annex K (page 1). Can you confirm that this information is indeed releasable? It seems that the claim of confidentiality is likely a standard statement provided by the contract laboratory and may not apply presently.

Regards,

Richard Bonnette

**Richard E. Bonnette, M.S.**  
Center for Food Safety and Applied Nutrition  
Office of Food Additive Safety  
U.S. Food and Drug Administration  
Tel: 240-402-1235  
[richard.bonnette@fda.hhs.gov](mailto:richard.bonnette@fda.hhs.gov)





**1. Procedure for determination of ash content (Reference: In-house)**

Sr. No	Test	Method
01	Procedure	<ul style="list-style-type: none"> <li>• A silica crucible is placed on an oven at <math>105 \pm 1^\circ\text{C}</math> for 30 minutes.</li> <li>• The crucible is removed with a tongue and placed in a desiccator for 10 min.</li> <li>• The empty crucible is weighed (W1).</li> <li>• Approximately 1 g of test sample is added and evenly distributed in the crucible.</li> <li>• The crucible with the test sample is weighed (W2).</li> <li>• The crucible is placed on a muffle furnace at <math>600^\circ\text{C} \pm 25^\circ\text{C}</math> for one hour.</li> <li>• The crucible is removed with a tongue and kept in an oven at <math>105 \pm 1^\circ\text{C}</math> for 15 minutes.</li> <li>• To cool down the crucible, it is placed in a desiccator for 10 minutes.</li> <li>• The crucible is weighed (W3).</li> </ul>
02	Calculation	$\text{Ash Contents (\% w/v)} = \frac{W3 - W1}{W2 - W1} \times 100$

## 2. Procedure for determination of loss on drying (Reference: In-house)

Sr. No	Test	Method
01	Procedure	<ul style="list-style-type: none"> <li>An empty weighing bottle is dried in an oven at <math>105 \pm 1^\circ\text{C}</math> for 30 minutes.</li> <li>The weighing bottle is cooled in a desiccator for 10 minutes and weighed (W1).</li> <li>Approximately 1 g of test sample is added to the bottle and weighed (W2).</li> <li>The substance is evenly distributed in a weighing bottle.</li> <li>The bottle with the sample is dried in an oven at <math>105 \pm 1^\circ\text{C}</math> for 60 minutes.</li> <li>The bottle is cooled down to room temperature for 10 minutes.</li> <li>The bottle is weighed (W3).</li> </ul>
02	Calculation	$\text{Loss on drying (\%)} = \frac{W2 - W3}{W2 - W1}$ <p>Weight of empty bottle: (W1)            Weight of empty bottle + sample: (W2)            Weight of bottle after drying: (W3)</p>

## 3. Procedure for determination of TOS (Reference: In-house)

Sr. No	Test	Method
01	Procedure	<p>The content of total organic solids (TOS) is calculated as follows:  <math>\% \text{ TOS} = 100 - (A + W + D)</math></p> <p>A = % Ash            W = % Loss on drying            D = % Diluents and / or other formulation ingredients, if present</p>

#### 4. Procedure for determination of protein content by Kjeldahl method (Reference: In-house)

Sr. No	Test	Method
01	Nitrogen content	<ul style="list-style-type: none"> <li>0.3 g of sample are weighed and transferred to the digestion flask.</li> <li>6 g of digestion mixture (100 g of sodium sulphate and 4 g of copper sulphate AR grade) and 20 ml of concentrate H<sub>2</sub>SO<sub>4</sub> are added.</li> <li>This mixture is digested by heating about 60 – 90 minutes (until blue or slight greenish blue colour is observed) and cooled.</li> <li>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.</li> <li>30% solution of NaOH is added to the digestion flask until a dark colour is observed.</li> <li>This flask is connected to the condenser with a water-cooling system.</li> <li>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.</li> <li><b>TEST:</b> 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).</li> <li><b>BLANK:</b> 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).</li> </ul>
02	Calculation	$\text{Nitrogen content (\%w/w)} = \frac{(B - A) \times 0.14}{\text{Weight of sample}}$
03	Protein Content	$\text{Protein Content (\%)} = \% \text{ of nitrogen content} \times 6.38$

9 pages have been removed in accordance with copyright laws. The removed reference citations are:

AOAC 18Edn 2005 (ICP), AOAC Official Method 999.10 Lead, Cadmium, Zinc, Copper, and Iron in Foods

AOAC 18Edn 2005 (ICP), AOAC Official Method 984.27 Calcium, Copper, Iron, Magnesium, Manganese, Phosphorus, Potassium, Sodium, and Zinc in Infant Formula

European Pharmacopoeia 5.6, 2.6.13. MICROBIOLOGICAL EXAMINATION OF NON-STERILE PRODUCTS: TEST FOR SPECIFIED MICRO-ORGANISMS

<https://uk.vwr-cmd.com/ex/downloads/datasheets/pheur/dok3.pdf>

## 7. Procedure for determination of antibacterial activity

*New specifications prepared at the 61st JECFA (2003) and published in FNP 52 Add. 11 (2003). An ADI "not specified" was established.*

### Antibacterial Activity

#### Scope

This procedure is designed for the determination of antibacterial activity in enzyme preparation derived from microbial sources.

#### Principle

The assay is based on the measurement of inhibition of bacterial growth under specific circumstances.

#### Culture Plates

Six organisms are tested: *Staphylococcus aureus* (ATCC 6538); *Escherichia coli* (ATCC 11229); *Bacillus cereus* (ATCC 2); *Bacillus circulans* (ATCC 4516); *Streptococcus pyrogenes* (ATCC 12344); and *Serratia marcescens* (ATCC 14041).

Make a test plate of each organism by preparing a 1:10 dilution of a 24 h Trypticase Soya Broth culture in Trypticase Agar (TSA) (for *Streptococcus pyrogenes* a 1:20 dilution).

Pour 15 ml of plain TSA into a Petri dish and allow the medium to harden. Overlay with 10 ml of seeded TSA and allow to solidify. Place a paper disk prepared according to Disk

Preparation of the tested enzyme on each of the six inoculated plates.

#### Disk Preparation

Make a 10% solution of the enzyme by adding 1 g of enzyme to 9 ml of sterile, distilled water.

Mix thoroughly with a Vortex mixer to obtain a homogeneous suspension. Autoclave suitable paper disks (for instance, S & S Analytical Filter Papers No. 740-E, 12.7 mm in diameter),

then saturate them with the enzyme by application of 0.1 ml (about 3 drops) of a 10% solution of the enzyme to the disk surface. Prepare six disks (one for each of the six

organisms) for each enzyme: place one disk on the surface of the six inoculated agar plates.

#### Incubation

Keep the six plates in the refrigerator overnight to obtain proper diffusion. Incubate the plates

at 37° for 24 h. Examine the plates for any inhibition zones that may have been caused by the enzyme preparation.

123

#### Interpretation

A visually clear zone around a disk (total diameter: 16 mm) indicates the presence of antibacterial components in the enzyme preparation. If an enzyme preparation shows obvious antibacterial activity against three (or more) organisms, it is concluded that antimicrobial agents are present.



## 8. Procedure for determination of mycotoxins

Return address: P.O. Box 844, 3700 AV Zeist, The Netherlands

Advanced Enzyme Technologies Ltd.  
Attn. Dr Anil Gupta  
Sun Magnetica, 'A' Wing, 5th Floor  
LIC Service Road, Louiswadi  
THANE (W) - 400 604  
INDIA

**Subject**  
Mycotoxine screening

Dear Dr Gupta,

Please find in the attachment information on the Triskelion used method for Mycotoxin screening. Due to reasons of IP and secrecy of the method it is not possible to provide further details.

With kind regards,

(b) (6)

Ton Schouten  
Study director  
Analytical Research Department

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

www.triskelion.nl

T +31 88 866 28 00  
F +31 88 866 87 28  
info@tno.triskelion.nl

**Date**  
16 September 2014

**Our reference**  
M&S/14/498

**E-mail**  
ton.schouten@tno.triskelion.nl

**Direct dialling**  
+31 88 866 1767

**Enclosure**  
Test results

General Terms and Conditions for Commissions to TNO Triskelion B.V., as filed at the Chamber of Commerce in Utrecht shall apply to all instructions given to TNO Triskelion B.V. The General Terms and Conditions will be sent on request

Registration number: 51382997

**The following method is used by Triskelion BV****Date**  
16 September 2014**Mycotoxin screening method.****Our reference**  
M&S/14/498

Samples (10 g) are extracted using an acetonitrile/water extraction solution (40 ml). After extraction for 1.5 hours, the solution is centrifuged and 4 x diluted. The extracts obtained are analyzed for the possible presence of mycotoxins by means of high performance liquid chromatography in combination with Quadruple MS-MS ESI (LC-MS) using "TNO Triskelion B.V. SOP MYC024". Quantification occurs by means of external standard solutions. Each time the analysis is run for each matrix a recovery test is carried out with spiked samples (low level and high level). In doing so, we collect validation data.

**Page**  
2/3

## Typical instrument settings:

Column:	Waters Atlantis T3, 5 $\mu$ m, 150 mm and 3 mm (or equivalent)
Flow:	0.3 ml/min
Injection volume:	20 $\mu$ l
Mobile phase:	methanol/ ammonium formiate gradient
Detection:	electrospray (ES)/ positive

MS settings:

Date  
16 September 2014

Our reference  
M&S/14/498

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Component	precursor ion	1st product ion
Deoxynivalenol	297	249
Nivalenol	313	175
Aflatoxine B1	313	241
Aflatoxine B2	315	259
Aflatoxine G1	329	243
Aflatoxine G2	331	245
Ochratoxine A	403,9	238,9
HT-2 Toxin	442	263
T-2 Toxin	484	305
Fumonisine B1	722,4	334,1
Zearalenone	319	186,9
Aflatoxine M1	328,9	273
Ergotamine	582	223,1
Ergocryptine	576	223
Ergocornine	562	223
Ergometrine	326	222,8
Ergocirstine	610	222,8
Ergosine	548	222,8

## ANNEX A2



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## STANDARD ANALYTICAL PROCEDURE

TITLE : LIPASE FCC BY FIP

Sr. No	Test	Method
--------	------	--------

01

**Principle** : This procedure is used to determine the lipase activity in preparations derived from microbial sources. The assay is based on the measurement of the amount of free fatty acids formed from an olive oil emulsion in the presence of sodium taurocholate over a fixed time interval. This assay is particularly used for measuring lipase activity in foods.

02

**Reagents** :

**1. Gum Arabic Solution:** Dissolve 110 g of gum arabic (acacia) (Sigma, Catalog No. G-9752, or equivalent) and 12.5 g of analytical-grade calcium chloride ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) in 800 mL of water in a 1000-mL volumetric flask, and dilute to volume with water. Shake or stir for 30 min at room temperature to dissolve completely. Centrifuge at  $4000 \times g$  for 20 min or filter through a Buchner funnel using Celite as a filter aid. Store the supernatant or filtrate at  $4^\circ$ . Divide into single-use, 24-mL aliquots. The solution is stable for 6 months at  $-20^\circ$ .

**2. Substrate Emulsion:** Place 130 mL of olive oil (Sigma, Catalog No. O-1500, or equivalent) and 400 mL of *Gum Arabic Solution* in a mixer bowl, and cool the mixture to  $5^\circ$  to  $10^\circ$  on ice. Emulsify the mixture with a Waring Blender, or equivalent, operated at high speed for 30 min, keeping the temperature below  $30^\circ$  by repeatedly mixing at high speed for 5 min and turning the blender off for 1 min. Check the quality of the emulsion microscopically: 90% of the droplets should have a diameter equal to or less than  $3 \mu\text{m}$ , and the remaining 10% should not exceed  $10 \mu\text{m}$ . The emulsion is stable for 3 days at  $4^\circ$ .

**3. Reference Standard Solution :** Dissolve an aliquot of Fungi Lipase-International FIP Standard (International Commission on Pharmaceutical Enzymes F.I.P., Center for Standards of the Federation Internationale Pharmaceutique, Harelbekestraat 72, B-9000 Gent, Belgium) in a 1% sodium chloride solution and dilute it to obtain a solution of 2.4 to 3.6 FIP microbial lipase units per milliliter. Prepare this solution fresh.

Prepared By	Checked & Approved By
QA Executive	QA Manager

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**STANDARD ANALYTICAL PROCEDURE**

**TITLE : LIPASE FCC BY FIP**

Sr. No	Test	Method
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**4. 0.02 N Sodium Hydroxide Solution:** Prepare daily by diluting 10 mL of analytical-grade 1 N sodium hydroxide to 500 mL with recently boiled water.  
**0.5% Sodium Taurocholate Solution** Dissolve 0.5 g of sodium taurocholate (DIFCO, Catalog No. 0278-15-8) in 100 mL of water. Prepare this solution fresh.

**03 Enzyme Preparation :** **Sample Preparation:** Dissolve an accurately weighed amount of the enzyme preparation in a 1% sodium chloride solution, and dilute to obtain a solution of 2.4 to 3.6 FIP microbial lipase units per milliliter. Prepare this solution fresh.

**04 Procedure :** [NOTE: Assay the Fungi Lipase-International FIP Standard as an internal standard each time.]  
**Automatic Titration:** Use an automatic titration device with a 25 mL ± 0.02 mL buret, a pH meter giving a resolution to 0.01, and a reaction vessel with a capacity of 100 mL. Add 24 mL of *Substrate Emulsion*, 9 mL of water, and 2 mL of *0.5% Sodium Taurocholate Solution* to the reaction vessel. Place the reaction vessel in a water bath preheated to 37° ± 0.5° over a hot plate provided with magnetic stirring, and add a magnet to the reaction vessel. Pre-incubate the reaction vessel at 37° ± 0.5° for 10 to 15 min while stirring at about 300 rpm. Immerse a pH-electrode and the tip of the buret into the solution. If desired, gently blow nitrogen gas onto the solution. Adjust the pH of the solution to 7.0 with *0.02 N Sodium Hydroxide Solution*. Set the automatic buret to zero. Add 5.0 mL of the enzyme solution while simultaneously starting a timer. Maintain the pH at 7.0 by automatic titration. After 10.0 min, abruptly (within 30 s) bring the pH to 9.0 by manually adding additional *0.02 N Sodium Hydroxide Solution*. Record the volume of *0.02 N Sodium Hydroxide Solution* consumed as  $N_1$ . Run the test with a blank by setting up the titration in the same manner, except after adjusting the pH to 7.0 with *0.02 N Sodium Hydroxide Solution*, set the automatic buret to zero, and maintain the pH at 7.0 by automatic titration. After 10.0 min, abruptly (within 30 s) bring the pH to 9.0 as before, and then add 5.0 mL of enzyme solution. Because the enzyme lowers

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**STANDARD ANALYTICAL PROCEDURE**

**TITLE : LIPASE FCC BY FIP**

Sr. No	Test	Method
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the pH, return the pH to 9.0 by adding 0.02 N *Sodium Hydroxide Solution*. Record the volume of 0.02 N *Sodium Hydroxide Solution* consumed as  $N_2$ .

**Manual Titration :** Follow the same procedure as with *Automatic Titration*, but keep the pH at 7.0 with 0.02 N *Sodium Hydroxide Solution* from a 25-mL buret, demarked in 0.02-mL units.

Calculation One unit of enzyme activity (FIP Unit) is defined as that quantity of a standard lipase preparation (Fungi Lipase-International FIP Standard) that liberates the equivalent of 1  $\mu\text{mol}$  of fatty acid per minute from the *Substrate Emulsion* under the described assay conditions. The specific activity is expressed in international FIP units per milligram of the *Sample Preparation*.

The use of an enzyme reference standard of known activity, controlled by the Center for Standards of the Commission, eliminates difficulties from interlaboratory differences in quality of reagents such as the *Gum Arabic Solution*, olive oil, or *Substrate Emulsion* or in the set-up of the experiment.

The activity (FIP U/mg) using an enzyme reference standard is calculated by the formula

$$(A \times C)/B$$

in which  $A$  is the specific activity, in units/mg, of the test sample (measured);  $B$  is the specific activity, in units/mg, of Fungi Lipase-International FIP Standard (measured); and  $C$  is the number of FIP units/mg of Fungi Lipase-International FIP Standard as indicated on the container.

One milliliter of the 0.02 N *Sodium Hydroxide Solution* corresponds with the neutralization of 20  $\mu\text{mol}$  of fatty acids. Five milliliters of enzyme solution liberates  $(N_1 - N_2) \text{ mL} \times 20 \mu\text{mol}$  of fatty acids over a 10-min time interval. If the enzyme solution contains  $W$  mg of enzyme preparation per milliliter, the specific activity, in units/mg, is calculated as follows:

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**STANDARD ANALYTICAL PROCEDURE**

**TITLE : LIPASE FCC BY FIP**

Sr. No	Test	Method
--------	------	--------

$$[(N_1 - N_2) \times 20] / (10 \times 5 \times W)$$

in which  $(N_1 - N_2)$  is the volume, in milliliters, of the 0.02 N Sodium Hydroxide Solution used for the titration.

Prepared By	Checked & Approved By
QA Executive	QA Manager

**1. Title: Technical Report on the Characterization of a *Rhizopus oryzae* Lipase (E.C.3.1.1.3) produced by a genetically modified *Aspergillus niger* strain (FL100SC)**

**Unique Study Code: ROL/AET/PRO/014A**

**Advanced Enzyme Technologies Ltd.**

Date: 25<sup>th</sup> February 2014

(b) (6)	(b) (6)
<b>Study Director</b>	<b>Study Monitor</b>
<p><b>Dr. Anil K. Gupta</b>  Advanced Enzyme Technologies Ltd.  A 61/62 Malegaon MIDC, Sinnar  Nashik-422113 India.  Email: anil@advancedenzymes.com</p>	<p><b>Dr. Stephanie A. Misquitta</b>  Advanced Enzyme Technologies Ltd.  “A” Wing, Sun Magnetica, 5<sup>th</sup> Floor,  LIC Service Rd., Louiswadi,  Thane (W), Pin 400 604, Maharashtra, India.  Email: stephanie@advancedenzymes.com</p>

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3. Summary

A lipase preparation from *Rhizopus oryzae* showed a molecular weight of ~ 35 kDa by size exclusion chromatography and 37.8 kDa by SDS-PAGE, indicating that it is a monomer. Zymogram analysis showed the presence of lipase activity corresponding to the protein band at ~ 37.8 kDa. Schiff's staining of the SDS-PAGE gel indicated that the enzyme is not glycosylated.

4. Quality Statement

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

Date: 25<sup>th</sup> February 2014

<p>(b) (6)</p> 	<p>(b) (6)</p> 
---	---

Dr. Anil K. Gupta  
Study Director

Dr. Stephanie A. Misquitta  
Study Monitor

### 5. Introduction

Lipases (Triacylglycerol acyl hydrolases E.C.3.1.1.3) are found in all living species of the animal kingdom, as well as plants and microorganisms such as yeast, bacteria, and fungi. Lipases are enzymes that catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. Lipases have considerable physiological significance and industrial potential. They are widely used in industrial applications such as the production of pharmaceuticals, cosmetics, leather, detergents, foods, perfumes, medical diagnostics, and other organic synthetic materials.

In this study, a lipase from *Rhizopus oryzae*, produced by the GM strain of *A. niger* (strain FL100SC) was characterized.

### 6. Study Title and Unique Study Code

Characterization of a lipase from *Rhizopus oryzae*

Unique Study Code: ROL/AET/PRO/014A

### 7. Study Objective

The objective of the study was to characterize lipase from *Rhizopus oryzae* in terms of its molecular weight, subunit composition, and glycosylation.

### 8. Study Location

Wagle Research Center

Advanced Enzyme Technologies Ltd.

### 9. Dates of the Study

May 2013 – February 2014

### 10. Details of the Enzyme Sample Used

Table 1. Details of the test product		
Product name	Manufacturer	Lot No., Manufacture date
Lipase	Advanced Enzyme Technologies Ltd.	Lot number: 0413134 Manufactured: 04/2013

### 11. Materials and Methods

#### 11.1. Characterization of the Enzyme

The molecular weight of the enzyme was determined using size exclusion chromatography and SDS-PAGE. The band having lipase activity (on SDS-PAGE gel) was determined by zymography. Schiff's staining was performed to determine if the enzyme is a glycoprotein.

#### 11.2. Size Exclusion Chromatography (SEC)

SEC was performed using a HiLoad 16/600 Superdex 200pg gel filtration column. A detailed procedure is given in Appendix 6.

#### 11.3. Analysis by SDS-PAGE

The enzyme sample was analysed on a 12 % SDS-PAGE gel. A detailed procedure is given in **Appendix 7**.

#### 11.4. Zymogram Analysis

The enzyme sample was electrophoresed on a 12 % gel. After electrophoresis, the gel was overlaid on a 1% agarose gel containing tributyrin (substrate) till a yellow coloured activity zone against a pink background was visible. A detailed procedure is given in Appendix 8.

#### 11.5. Glycoprotein Staining Using Schiff's Reagent



The enzyme sample was subjected to SDS-PAGE, after which the gel was developed for staining of glycoproteins using Schiff's reagent. Horseradish peroxidase (HRP) was used as the positive control. A detailed procedure is given in Appendix 9.

## 12. Result

### 12.1. Size Exclusion Chromatography (SEC)

The molecular weight obtained from SEC is ~35 kDa. Further determination of the molecular weight was carried out by SDS-PAGE.

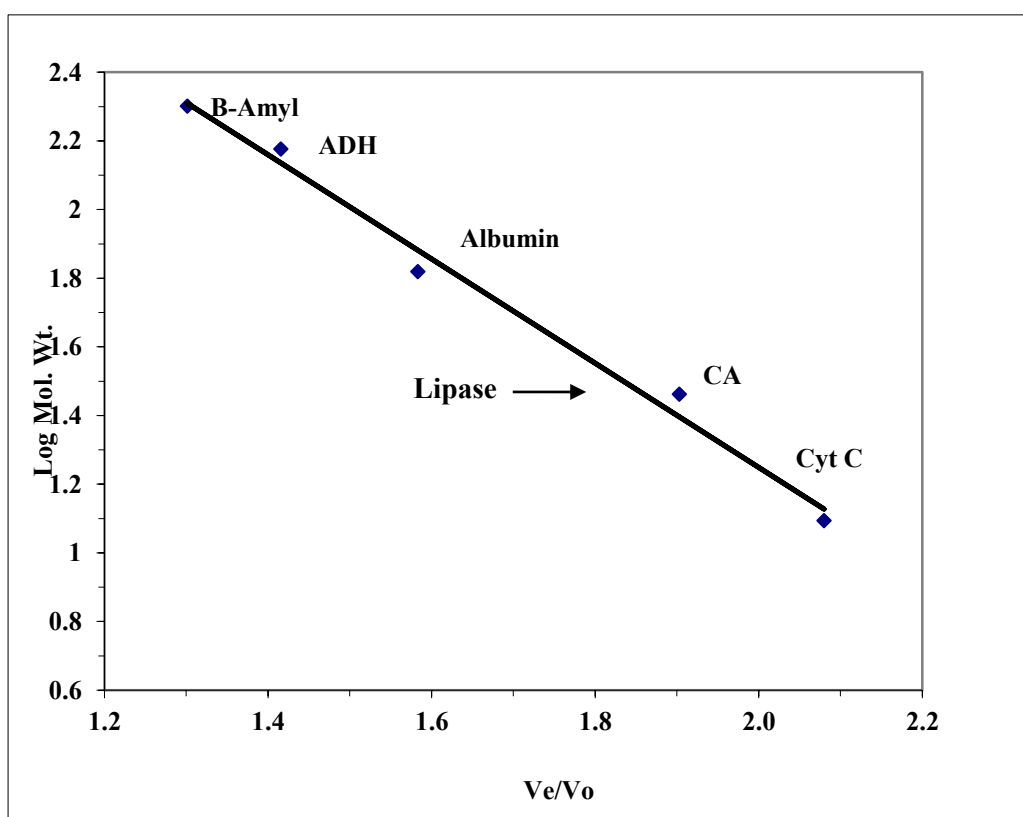


Figure 1. Size Exclusion Chromatography of Lipase

12.2. Analysis by SDS-PAGE

Based on SDS-PAGE, the molecular weight of the lipase is 37.8 kDa.

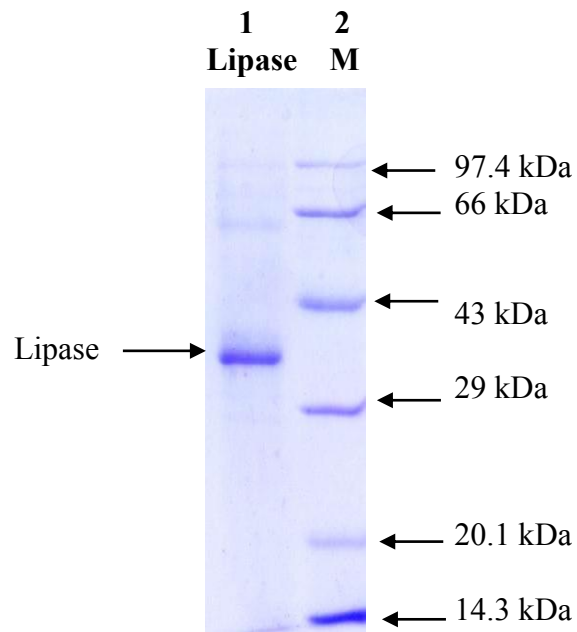


Figure 2. SDS-PAGE of Lipase

**Lane 1:** Lipase

**Lane 2:** M – Protein Markers

12.3. Zymogram Analysis

A yellow coloured activity zone corresponding to ~ 38 kDa, against a pink background was obtained, indicating that this protein band is a lipase.

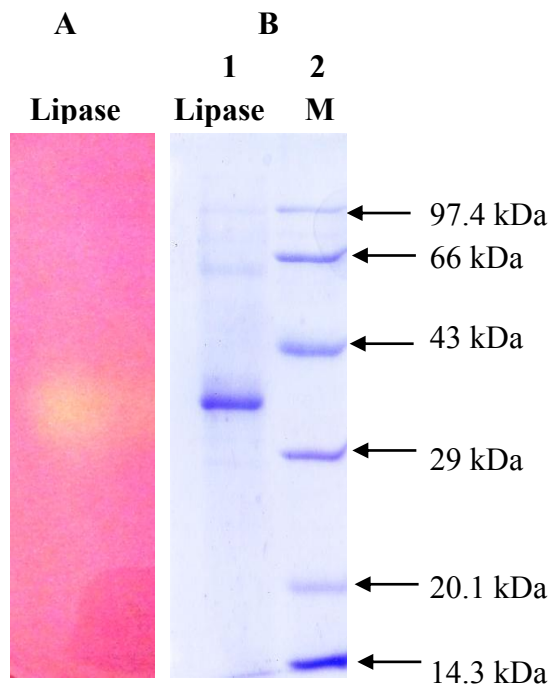


Figure 3. Zymogram of Lipase

**Fig A:** Zymogram for lipase

**Fig B:** Coomassie Staining

**Lane 1:** Lipase

**Lane 2:** M - Protein Markers

12.4. Glycoprotein Staining Using Schiff's Reagent

No magenta band was obtained for lipase at 37.8 kDa, indicating that the enzyme is not glycosylated.

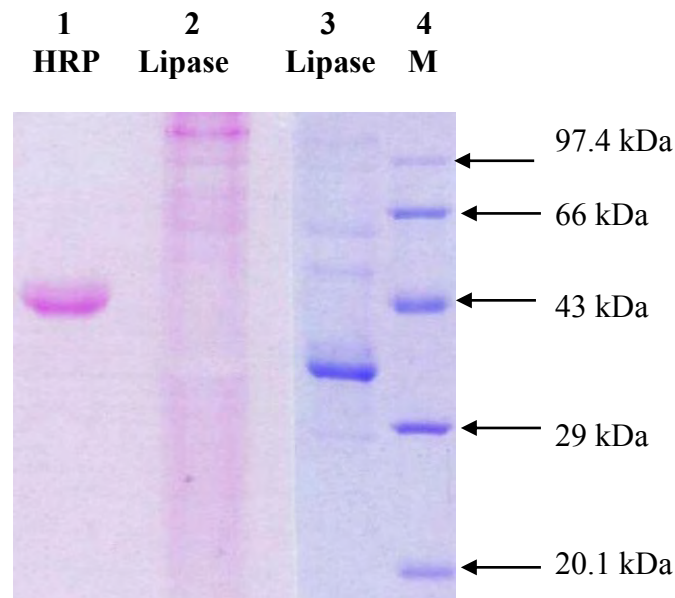


Figure 4. Glycoprotein Staining of Lipase

**Lane 1 - 2:** Glycoprotein Staining

Lane 1: HRP -Horseradish peroxidase (positive control)

Lane 2: Lipase

**Lanes 3 - 4:** Coomassie Staining

Lane 3: Lipase

Lane 4: M - Protein Markers

### *13. Discussion*

The lipase from *Rhizopus oryzae* characterized in this study has a molecular weight of 37.8 kDa. Results from both SEC and SDS-PAGE indicate that it is a monomer. Zymogram analysis indicated the presence of a lipase of size, 37.8 kDa. Based on Schiff's staining of an SDS-PAGE gel, our results indicate that the lipase is not glycosylated.

### *14. Conclusion*

The lipase from *Rhizopus oryzae*, produced by a GM strain of *A. niger* (strain FL100SC), was characterized in this study. Based on SDS-PAGE, and SEC, the enzyme is a monomer with a MW of 37.8 kDa. Zymogram analysis further confirmed that the protein of molecular weight, 37.8 kDa is indeed a lipase. Staining with Schiff's reagent, indicated that the enzyme is not a glycoprotein.

*15. Bibliography*

- Hiol, A., Jonzo, M. D., Rugani, N., Druet, D., Sarda, L., & Comeau, L. C. (2000). Purification and characterization of an extracellular lipase from a thermophilic *Rhizopus oryzae* strain isolated from palm fruit. *Enzyme and Microbial Technology*, 26, 421-430.
- Singh, R., Gupta, N., Goswami, V. K., & Gupta, R. (2006). A simple activity staining protocol for lipases and esterases. *Appl Microbiol Biotechnol.*, 70, 679-682.

*16. List of Appendices*

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*Appendix 2 – Certificate of Analysis of Lipase Used*

*Appendix 3 – List of Abbreviations*

*Appendix 4 – List of Reagents*

*Appendix 5 – List of Instruments*

*Appendix 6 – Procedure for Size Exclusion Chromatography*

*Appendix 7 – Procedure for SDS-PAGE*

*Appendix 8 – Procedure for the Zymogram of Lipase*

*Appendix 9 – Procedure for Glycoprotein Staining Using Schiff's Reagent*



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

**Appendix 2 - Certificate of Analysis of Lipase Used**

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

**QUALITY ASSURANCE DEPARTMENT****CERTIFICATE OF ANALYSIS**

**PRODUCT NAME** : RHIZOPUS LIPASE  
**BATCH NO.** : 0413134  
**MFG. DATE** : APRIL,2013  
**EXPIRY DATE** : MARCH, 2015

**PROTOCOL OF ANALYSIS**

TEST	RESULT	LIMITS
<b>Description</b>	Light Brown coloured amorphous, hygroscopic powder; having characteristics odour.	Light Brown to Brown coloured amorphous, hygroscopic powder; having characteristics odour.
<b>Solubility</b>	Soluble in Water : <b>Complies</b>	Soluble in Water.
<b>Lead</b>	: <b>Complies</b>	Not more than 5 ppm
<b>Microbial Limit- Total viable count Total coliforms/g Escherichia.coli/25g Salmonellae/25g</b>	: <b>Complies</b> : <b>Complies</b> : <b>Complies</b> : <b>Complies</b>	NMT $1 \times 10^4$ cfu/g Not more than 30 Negative by test Negative by test
<b>Antimicrobial Activity</b>	Absent by test : <b>Complies</b>	Absent by test
<b>Rhizopus Lipase Activity</b>	101,254 FIP U/g	NLT 100,000 FIP U/g

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

(b) (6)

QA-CHEMIST  
 Date: APRIL 24, 2013

(b) (6)

MANAGER-QUALITY ASSURANCE

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Head Office : Sun Magnetica, 'A' wing, 5th Floor, LIC Service Road, Louiswadi, Thane (W) - 400 604, India. Tel.: +91-22-4170 3200, Fax: +91-22-2583 5159

**Appendix 3 - List of Abbreviations**

<b>Abbreviation</b>	<b>Full Name</b>
APS	Ammonium persulphate
°C	degree Celsius
CV	Column volume
g	grams
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
kDa	Kilo Dalton
M	Molar
mg	milligrams
mg/ml	milligrams per millilitre
ml	millilitre
ml/min	millilitre per minute
µg	microgram
µm	micrometre
MW	molecular weight
nm	nanometer
N	Normal
%	percentage
rpm	Revolutions per minute
SEC	Size Exclusion Chromatography
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TEMED	Tetramethylethylenediamine
w/v	weight by volume
v/v	volume by volume

**Appendix 4 - List of Reagents**

Sr. No.	Reagent Name	Source	Catalogue No.
1.	Acetic acid glacial	Merck Specialities Pvt. Ltd.	60006305001730
2.	Agarose powder medium ecco	SRL Pvt. Ltd.	0140114
3.	Ammonium persulphate	SRL Pvt. Ltd.	0148134
4.	Beta mercaptoethanol	Sigma - Aldrich	M3148
5.	Bis-acrylamide	SRL Pvt. Ltd.	0140331
6.	Bromophenol blue	SRL Pvt. Ltd.	0240168
7.	Butanol	Merck Specialities Pvt. Ltd.	82226205001730
8.	Coomassie brilliant blue R250	SRL Pvt. Ltd.	024018
9.	Disodium phosphate	Sigma - Aldrich	S5136
10.	Ethanol	Changshu Yangyuan Chemical	XK-13-201-00185
11.	Gel Filtration Markers	Sigma - Aldrich	MWGF200
12.	Glyceryl tributyrate	Sigma - Aldrich	T8626
13.	Glycerol	Merck Specialities Pvt. Ltd.	61756005001730
14.	Glycine	Merck Specialities Pvt. Ltd.	3570-500GMCN
15.	Horseradish peroxidase	Sigma - Aldrich	P6782
16.	Hydrochloric acid, min 35%	Merck Specialities Pvt. Ltd.	61762505001730
17.	Methanol	Merck Specialities Pvt. Ltd.	6060072500
18.	Periodic acid	Sigma - Aldrich	P7875
19.	Phenol Red indicator	BDH Chemicals	37913
20.	Protein molecular weight markers (medium range)	Merck Specialities Pvt. Ltd.	623110275001730
21.	Schiff's reagent	Sigma - Aldrich	S5133
22.	Sodium chloride	Merck Specialities Pvt. Ltd.	60640405001730

<b>Sr. No.</b>	<b>Reagent Name</b>	<b>Source</b>	<b>Catalogue No.</b>
23.	Sodium dodecyl sulphate	Sigma - Aldrich	L3771
24.	Sodium hydroxide	Sigma - Aldrich	221465
25.	Sodium metabisulphite	S D Fine-Chem Ltd.	40180
26.	TEMED	Merck Specialities Pvt. Ltd.	623171280051730
27.	Tris Base	Merck Specialities Pvt. Ltd.	613600701001730
28.	Triton X-100	S D Fine-Chem Ltd.	40632

**Appendix 5 - List of Instruments**

<b>Sr. No.</b>	<b>Name of Instrument</b>	<b>Source</b>	<b>Instrument No.</b>
1.	AKTA Basic	GE Healthcare	WRC/PRO-001
2.	Frac-900	GE Healthcare	WRC/PRO-002
3.	Spectrophotometer (UV-Vis-650)	Jasco	WRC/PRO-003
4.	Mini gel electrophoresis unit	Tarsons	WRC/PRO-005
5.	Watson Marlow pump	Watson	WRC/PRO-006
6.	Heating block	Neolab	WRC/PRO-008 WRC/PRO-017
7.	Gel rocker	Neolab	WRC/PRO-009
8.	Bench top centrifuge (RM-12CDX)	Remi	WRC/PRO-012
9.	Incubator	Remi	WRC/MBL-013
10.	Magnetic stirrer	Galaxy	WRC/ANA-006
11.	pH meter	Labindia	WRC/ANA-005
12.	Hot plate	Adinath	WRC/ANA-013
13.	Balance	AND	WRC/ANA-001
14.	Vortex	SPINIX	WRC-MBL-018

## **Appendix 6 - Procedure for Size Exclusion Chromatography**

**Principle:** Size exclusion chromatography or gel filtration is a technique that separates proteins in their native state, according to size.

### **1. Column Required:**

- i) HiLoad 16/600 Superdex 200 pg (GE Healthcare)

### **2. Reagents Required:**

- i) Buffer: 0.05 M Sodium Phosphate + 0.15 M NaCl, pH 7.0
- ii) 1 N Hydrochloric acid
- iii) 20 % (v/v) Ethanol
- iv) Milli-Q water
- v) Gel filtration markers kit for protein molecular weight 12 kDa - 200 kDa: Cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (BSA) (66 kDa), alcohol dehydrogenase (150 kDa),  $\beta$ -Amylase (200 kDa), and blue dextran (2,000 kDa).

**3. Sample Preparation:** Dissolve an appropriate amount of sample in the buffer, filter it through a 0.22  $\mu$ m filter or centrifuge at 10,000 rpm for 10 minutes.

### **4. Procedure:**

- i) Equilibrate the column with 2 CVs (column volumes) of buffer.
- ii) Load the sample, collect 1 ml fractions.

### **References:**

As per the manufacturer's instructions.



## **Appendix 7 - Procedure for Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

**Principle:** SDS-PAGE separates proteins according to their size. During electrophoresis, a mixture of proteins is 'loaded' into a lane in the gel. Under the influence of an electric current, these proteins are separated based on their mass. The individual proteins bands can be visualised after staining (Coomassie/ silver ). By using protein standards of known molecular weights, the molecular weight of the protein of interest can be determined.

### **1. Reagents Required:**

- i) 0.025 M Tris + 0.2 M Glycine + 0.1 % SDS (1X Running Buffer), pH 8.8
- ii) Resolving buffer: 1.5 M Tris-HCl, pH 8.9
- iii) Stacking buffer: 0.5 M Tris-HCl, pH 6.8
- iv) 10 % (w/v) SDS
- v) 30 % (w/v) Acrylamide / Bisacrylamide (29:1) Solution
- vi) TEMED
- vii) 10 % (w/v) APS
- viii) 6X Sample Buffer

### **2. Preparation of SDS-PAGE Tris-Glycine Gel:**

#### **2.1. Protocol:**

Cast the gels as per the requirement. (See the table below).

##### **2.1.1 Resolving Gel 10 % & Stacking Gel 4 %**

<b>Sr. No.</b>	<b>Reagents</b>	<b>Resolving Gel: Volume (ml)</b>	<b>Stacking Gel: Volume (ml)</b>
1.	Acrylamide/Bisacrylamide solution	2.0	0.8
2.	1.5 M Tris-HCl buffer, pH 8.8	1.5	---
3.	0.5 M Tris-HCl buffer, pH 6.8	---	1.5
4.	Distilled water	2.4	3.64
5.	10% SDS	0.06	0.06
6.	TEMED	0.006	0.006
7.	10% APS (ml)	0.06	0.06

#### **2.2. Electrophoresis:**

- i) Perform the electrophoresis at 150 volts.
- ii) After completion, wash the gel 2 to 3 times with distilled water. Stain with Coomassie.

## **Appendix 8 - Procedure for the Zymogram of Lipase**

**Principle:** This method is used to detect the protein band containing lipase activity in the polyacrylamide gel. After separation of proteins by SDS-PAGE, the gel is allowed to react with the substrate under suitable conditions. Lipase in the gel hydrolyzes the substrate resulting in the release of fatty acids which decreases the pH. This decrease in pH is detected using a pH indicator and is visible as a yellow coloured band against a pink background.

### **1. Reagents:**

#### **For the Zymogram**

- i) 2.5 % (w/v) Triton X-100
- ii) 0.05 M Sodium phosphate buffer, pH 8.0
- iii) 1 N Sodium hydroxide

#### **For SDS-PAGE:**

(For the procedure of SDS-PAGE refer to 'Error! Reference source not found.').

### **2. Reagent Preparation:**

#### **Preparation of the substrate gel**

1 % w/v glyceryl tributyrates + 1 % w/v agarose + phenol red, adjust the pH to 8.0 using 1 N sodium hydroxide.

### **3. Procedure:**

- i) After electrophoresis incubate the gel in 2.5 % (v/v) Triton X-100 for 1 hour.
- ii) After incubation in Triton X-100, wash the gel with 0.05 M phosphate buffer, pH 8.0 for 30 minutes.
- iii) Overlay the polyacrylamide gel on the substrate gel, incubate for 4 hours at 40 - 45°C. (Note: Preferably incubate till the yellow coloured activity zone is observed against the pink coloured background.)

### **References:**

Singh, R., Gupta, N., Goswami, V. K., & Gupta, R. (2006). A simple activity staining protocol for lipases and esterases. *Appl Microbiol Biotechnol.*, 70, 679-682.

### **Appendix 9 - Procedure for Glycoprotein Staining Using Schiff's Reagent**

**Principle:** The method is used to detect sugar moieties of glycoproteins on a gel, after electrophoresis. The reaction between the reagent and the glycosyl moieties yields magenta bands.

#### **1. Reagents Required:**

- i) Schiff's reagent
- ii) Horseradish peroxidase (HRP): To be used as a positive control
- iii) 1 % (w/v) Periodic acid + 3 % Acetic acid
- iv) 0.58 % (w/v) Sodium metabisulphite
- v) 40 % (v/v) Methanol

#### **2. Procedure:**

After electrophoresis, stain the gel as follows.

(Perform the following procedure in a well ventilated area / hood).

- i) After electrophoresis, fix the gel with 40 % methanol, for one hour. Wash with distilled water
- ii) Immerse the gel in 1% periodic acid – 3% acetic acid solution, for 30 minutes. Wash the gel with distilled water, twice, 20 minutes each time.
- iii) Immerse the gel in Schiff's reagent in the dark, till magenta coloured bands are observed.
- iv) Once the bands appear, wash the gel in 0.58% metabisulphite solution, till the faint pink colour of the background is eliminated.

#### **References:**

As per the manufacturer's (Sigma-Aldrich) instructions.

**1. Title: Technical Report on the Characterization of a *Rhizopus oryzae* Lipase (EC 3.1.1.3) produced by a genetically modified *Aspergillus niger* strain (FL100SC)**

**Unique Study Code: ROL/AET/PRO/014B**

**Advanced Enzyme Technologies Ltd.**

Date: 24<sup>th</sup> June, 2014

(b) (6)	(b) (6)
<b>Study Director</b> <b>Dr. Anil K. Gupta</b> Advanced Enzyme Technologies Ltd. A 61/62 Malegaon MIDC, Sinnar Nashik-422113 India. Email: anil@advancedenzymes.com	<b>Study Monitor</b> <b>Dr. Stephanie A. Misquitta</b> Advanced Enzyme Technologies Ltd. "A" Wing, Sun Magnetica, 5 <sup>th</sup> Floor, LIC Service Rd., Louiswadi, Thane (W), Pin 400 604, Maharashtra, India. Email: stephanie@advancedenzymes.com

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### 3. Summary

A lipase from *Rhizopus oryzae* produced by a genetically modified *Aspergillus niger* strain (FL100SC) was characterized in this study. The objective of the study was to determine the influence of temperature, pH, inhibitors and activators on the activity and stability of the lipase. Results from this study show that for this enzyme, the optimum pH is 7.5, and the optimum temperature is 45°C. The enzyme is stable up to 40°C for 2 hours. At 25°C, it is stable in the range of pH 5.0 - 9.0 for 2 hours. Lipase activity is strongly inhibited by Zn<sup>2+</sup>, Cu<sup>2+</sup>, Co<sup>2+</sup> and Hg<sup>2+</sup>, partially inhibited by Na<sup>+</sup>, K<sup>+</sup>, Mg<sup>2+</sup> and EDTA. The enzyme is partially activated by Ca<sup>2+</sup>.

### 4. Quality Statement

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

Date: 24<sup>th</sup> June, 2014

<p>(b) (6)</p> 	<p>(b) (6)</p> 
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Dr. Anil K. Gupta  
Study Director

Dr. Stephanie A. Misquitta  
Study Monitor

### 5. Introduction

Lipases (EC 3.1.1.3) are found in all types of living organisms from fungi and bacteria to plants and animals. Lipases hydrolyse the ester bonds in triglycerides, to form fatty acids and glycerol. For the characterization of this enzyme, the enzyme activity was measured titrimetrically, using olive oil as the substrate. This method is based on the lipase assay method published in the Food Chemical Codex (FCC, Sixth Edition, 2008-2009)

### 6. Study Title and Unique Study Code

Technical Report on the Characterization of a *Rhizopus oryzae* Lipase (E.C.3.1.1.3) produced by a genetically modified *Aspergillus niger* strain (FL100SC).

Unique Study Code: ROL/AET/PRO/014B.

### 7. Study Objective

The objective of the study was to determine the influence of temperature and pH on lipase activity and stability, and to identify the inhibitors and activators of the enzyme.

### 8. Study Location

Wagle Research Center

Advanced Enzyme Technologies Ltd.

### 9. Dates of the Study

Dec, 2013 - June, 2014

### 10. Details of the Enzyme Sample Used

Table 1. Details of the Test Product			
Product name	Manufacturer	Lot No., Manufacture Date	Activity (U/g)
Rhizopus Lipase	Advanced Enzyme Technologies Ltd.	Lot number: 0413134 Manufactured: April, 2013 Expiry: March, 2015	101,254
Definition of Unit Activity One unit of enzyme activity (FIP Unit) is defined as that quantity of a standard lipase preparation that liberates the equivalent of 1 $\mu\text{mol}$ of fatty acid per minute from the substrate emulsion under the described assay conditions.			



## *11. Materials and Methods*

### *11.1. Physical Parameters Measured*

The effect of pH and temperature on lipase activity and stability was studied by incubating the enzyme at the defined set of pH and temperature conditions. The effect of metal salts on enzyme activity was determined. The assay used to characterize the enzyme, is based on the standard method given in 'Food Chemicals Codex' (2008-2009, Sixth Edition). This is a titration method, using olive oil as the substrate.

### *11.2. pH Optimum of the Lipase*

Enzyme activity was measured in the pH range, 5.0 to 10.0 and at 37°C, using olive oil as the substrate. A detailed procedure is included in Appendix 6.

### *11.3. pH Stability of the Lipase*

To evaluate the stability of the lipase at different pH, the enzyme sample was incubated in buffer solutions at different pH (3.0 to 10.0) at 25°C for 2 hours. At the end of 2 hours, aliquots of the enzyme were withdrawn and the enzyme activity determined at pH 7.0 and 37°C using olive oil as the substrate. A detailed procedure is included in Appendix 7.

### *11.4. Temperature Optimum of the Lipase*

Enzyme activity was measured at different temperatures ranging from 25°C to 60°C using olive oil as the substrate, at pH 7.0. A detailed procedure is included in

Appendix 8.

#### *11.5. Temperature Stability of the Lipase*

To evaluate the stability of the lipase at different temperatures, the enzyme sample was incubated at different temperatures ranging from 25°C to 55°C for 2 hours. At the end of 2 hours, aliquots of the enzyme were withdrawn, and the enzyme activity was measured using olive oil as the substrate. A detailed procedure is included in Appendix 9.

#### *11.6. Effect of Inhibitors and Activators*

The activity was measured in the presence of metal salts and EDTA at 1 mM concentration, at pH 7.0 and 37°C using olive oil as the substrate. A detailed procedure is included in Appendix 10.

## 12. Results

### 12.1. pH Optimum of the Lipase

The activity of the enzyme at different pH, at 37°C, is given in Table 2. The maximum activity was observed at pH 7.5 and was assigned 100 %. The optimum pH range for lipase activity was 7.5 -8.0. Over 90% of lipase activity was retained in this pH range.

pH	Relative activity (%)
5	10.4
6	7.8
7	40.4
7.5	100.0
8	96.1
8.5	46.1
9	24.2
9.5	15.2
10	0.0

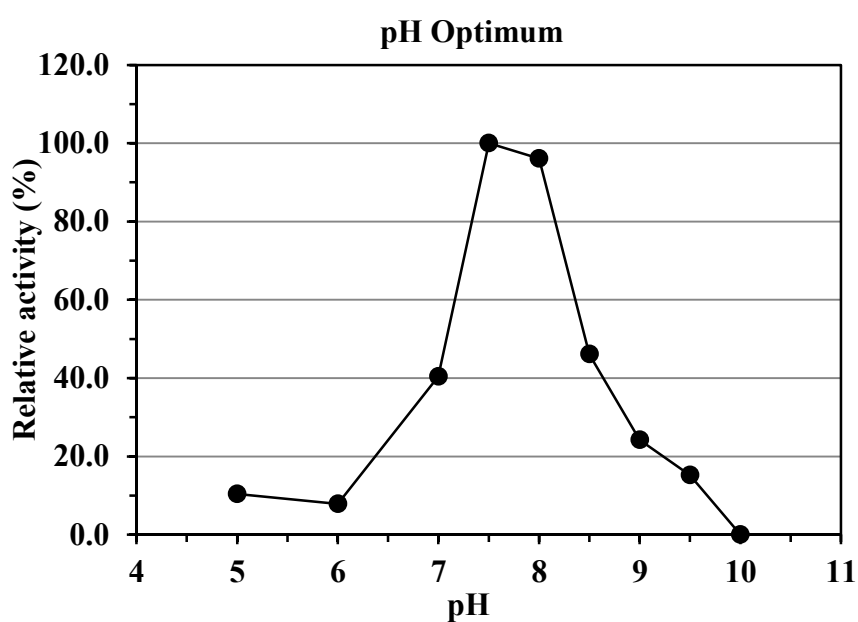


Figure 1. pH Optimum of the Lipase

12.2. pH Stability of the Lipase

The percentage of residual activity after 2 hours at different pH, is given in Table 3. The enzyme was found to be stable in the pH range, 5.0 - 9.0 at 25°C.

pH	Residual activity (%) after 2 hours
3	53.5
4	74.4
5	80.2
6	81.4
7	80.2
8	80.2
9	81.4
10	74.4

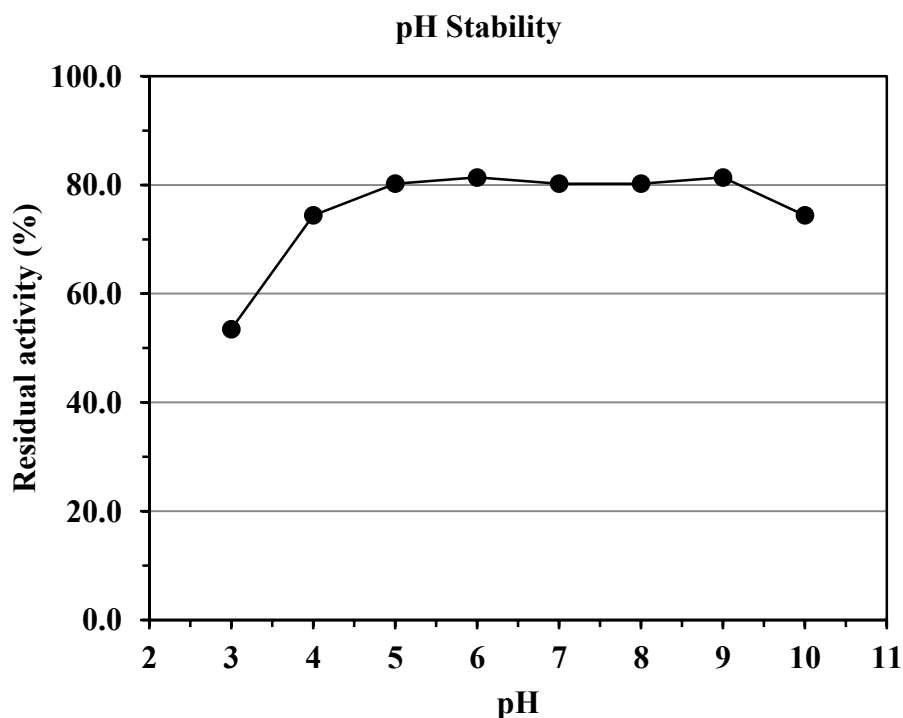


Figure 2. pH Stability of the Lipase

### 12.3. Temperature Optimum of the Lipase

The relative activity of the enzyme at pH 7.0 and at different temperatures is included in Table 4. Maximum activity was recorded at 45°C and assigned as 100%. The optimum temperature range for lipase activity was found to be 40 - 45°C.

Temperature (°C)	Relative activity (%)
25	27.0
30	57.1
35	73.0
40	87.3
45	100.0
50	60.3
55	28.6
60	3.2

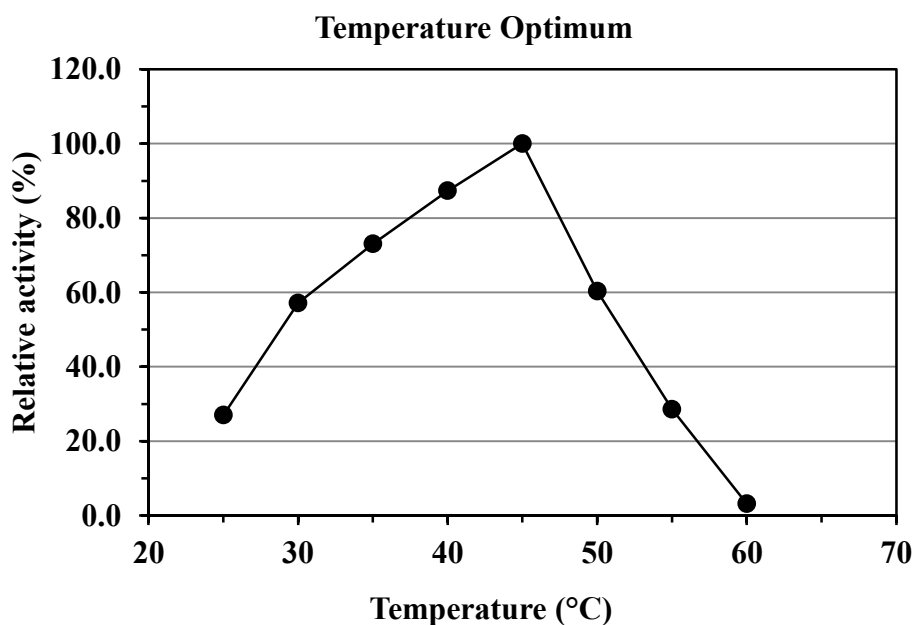


Figure 3. Temperature Optimum of the Lipase

12.4. *Temperature Stability of the Lipase*

The percentage of residual activity after 2 hours at different temperatures is included in Table 5. The enzyme was stable upto 40°C. At temperatures higher than 45°C, the enzyme was inactivated rapidly.

Table 5. Temperature Stability of the Lipase	
Temperature (°C)	Activity after 2 hours (%)
25	81.5
30	74.1
35	61.1
40	59.3
45	38.9
50	6.5
55	0.0

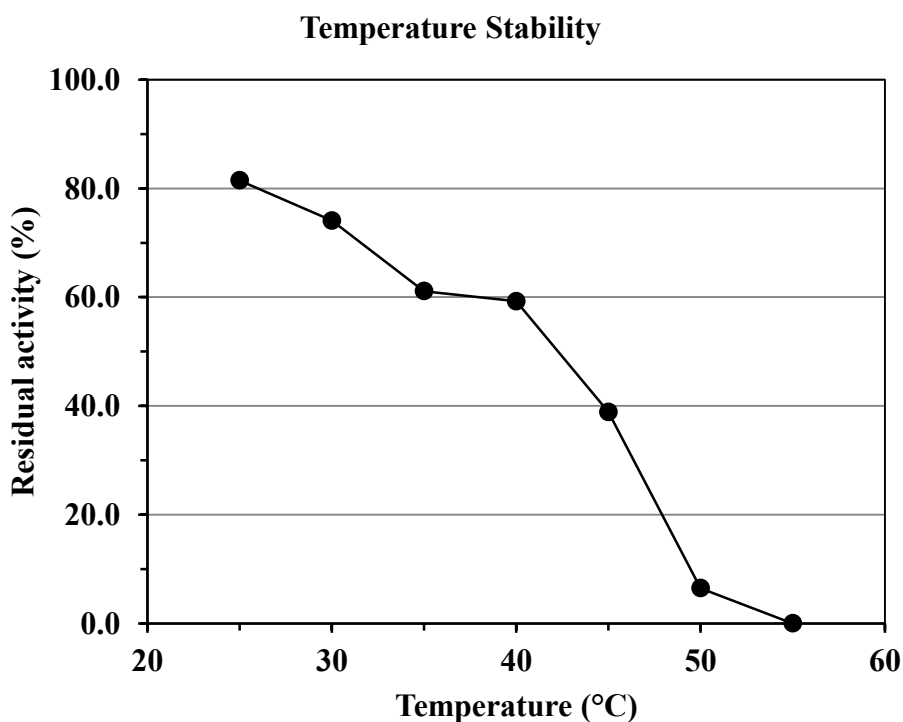


Figure 4. Temperature Stability of the Lipase

*12.5. Effect of Inhibitors and Activators*

The effect of various metal ions (at 1 mM concentration) on lipase activity was evaluated. The results are included in Table 6. Strong inhibition was observed in the presence of  $Zn^{2+}$ ,  $Cu^{2+}$ ,  $Hg^{2+}$ ,  $Co^{2+}$  and partial inhibition was observed in the presence of  $Na^+$ ,  $K^+$ ,  $Mg^{2+}$  and EDTA.  $Ca^{2+}$  partially activated the enzyme.

<b>Inhibitor/ Activator (1mM)</b>	<b>Residual activity (%)</b>
NaCl	80.8
ZnSO <sub>4</sub>	0.0
CuCl <sub>2</sub>	3.8
KCl	88.5
MgCl <sub>2</sub>	84.6
CoCl <sub>2</sub>	53.8
CaCl <sub>2</sub>	103.8
HgCl <sub>2</sub>	26.9
MnCl <sub>2</sub>	96.2
EDTA	61.5

*13. Discussion*

Maximum enzyme activity for the lipase was found to be between pH 7.5 - 8.0. Over 90% of lipase activity was retained in this pH range. The optimum temperature range for lipase activity was 40 - 45°C. The enzyme was found to be stable in the pH range of 5.0 - 9.0 at 25°C for 2 hours. Stability of lipases in this pH range has been reported in the literature (Wang, 2014). The lipase was stable in the temperature range of 25 - 40°C for 2 hours. Partial activation was observed by  $Ca^{2+}$ , whereas enzyme activity was strongly inhibited by  $Zn^{2+}$ ,  $Cu^{2+}$ ,  $Hg^{2+}$ ,  $Co^{2+}$  and partially inhibited by  $Na^+$ ,  $K^+$ ,  $Mg^{2+}$  and EDTA. A similar result was reported in a previous study (Hiol, 2000).



#### *14. Conclusion*

Results from this study show that for this lipase, the optimum pH is 7.5-8.0, and the optimum temperature is 45°C. Furthermore, the enzyme preparation is proven to be stable for 2 hours at 25°C in the pH range of 5.0 - 9.0 and the temperature range of 25 - 40°C. However, inhibition is seen in presence of  $Zn^{2+}$ ,  $Cu^{2+}$ ,  $Hg^{2+}$ ,  $Co^{2+}$ ,  $Na^+$ ,  $K^+$ ,  $Mg^{2+}$  and EDTA.

15. Bibliography

- ‘Food Chemicals Codex’, Sixth Edition. (2008-2009). *Lipase (Microbial) Activity for Medium- and Long-Chain Fatty Acids*, pp. 1127-1128.
- Hiol, A., Jonzo, M. D., Rugani, N., Druet, D., Sarda, L., & Comeau, L. C. (2000). Purification and characterization of an extracellular lipase from a thermophilic *Rhizopus oryzae* strain isolated from palm fruit. *Enzyme and Microbial Technology*, 26, pp. 421– 430.
- Wang, J.-R., Li, Y.-Y., Xu, S.-D., Li, P., Liu, J.-S., & Liu, D.-N. (2014). High-Level Expression of Pro-Form Lipase from *Rhizopus oryzae* in *Pichia pastoris* & its Purification and characterization. *International Journal of Molecular Sciences*, 203-217.

*16. List of Appendices*

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

*Appendix 2 – Certificate of Analysis of the Enzyme Sample Used*

*Appendix 3 – List of Abbreviations*

*Appendix 4 – List of Reagents*

*Appendix 5 – List of Instruments*

*Appendix 6 – Procedure for the Determination of Optimum pH*

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*Appendix 9 – Procedure for the Determination of Enzyme Stability at Different Temperatures*

*Appendix 10 – Procedure for the Determination of Inhibitors and Activators*

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

**Appendix 2: Certificate of Analysis of the Enzyme Used**



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

**QUALITY ASSURANCE DEPARTMENT**

**CERTIFICATE OF ANALYSIS**

**PRODUCT NAME : RHIZOPUS LIPASE**  
**BATCH NO. : 0413134**  
**MFG. DATE : APRIL,2013**  
**EXPIRY DATE : MARCH, 2015**

**PROTOCOL OF ANALYSIS**

TEST	RESULT	LIMITS
<b>Description</b>	Light Brown coloured amorphous, hygroscopic powder; having characteristics odour.	Light Brown to Brown coloured amorphous, hygroscopic powder; having characteristics odour.
<b>Solubility</b>	Soluble in Water : <b>Complies</b>	Soluble in Water.
<b>Lead</b>	: <b>Complies</b>	Not more than 5 ppm
<b>Microbial Limit- Total viable count Total colifo:ms/g Escherichia.coli/25g Salmonellae/25g</b>	: <b>Complies</b> : <b>Complies</b> : <b>Complies</b> : <b>Complies</b>	NMT 1 x 10 <sup>4</sup> cfu/g Not more than 30 Negative by test Negative by test
<b>Antimicrobial Activity</b>	Absent by test : <b>Complies</b>	Absent by test
<b>Rhizopus Lipase Activity</b>	101,254 FIP U/g	NLT 100,000 FIP U/g

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

(b) [Redacted]  
 QA-CH(E)MIST  
 Date:APRIL 24, 2013

(b) (6) [Redacted]  
 MANAGER-QUALITY ASSURANCE

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**Appendix 3: List of Abbreviations**

<b>Abbreviations</b>	<b>Full name</b>
°C	Degree Celsius
g	Grams
ml	Milliliter
M	Molar
mM	Millimolar
µm	Micrometer
µmoles	Micromole
N	Normal
%	Percentage
rpm	Revolutions per minute

**Appendix 4: List of Reagents**

Sr. No.	Reagent name	Source	Catalogue No.
1.	Calcium chloride dihydrate	Merck Specialities Pvt. Ltd.	10238005001046
2.	Citric acid anhydrous	Merck Specialities Pvt. Ltd.	61799705001730
3.	Copper (II) chloride dihydrate	Sigma-Aldrich	459097
4.	Cobalt (II) chloride hexahydrate	Sigma-Aldrich	C8661
5.	Disodium hydrogen phosphate dodecahydrate	Merck Specialities Pvt. Ltd.	20248 K05
6.	Disodium hydrogen phosphate dihydrate	Merck Specialities Pvt. Ltd.	61790905001730
7.	EDTA dihydrate	Sigma-Aldrich	E4884
8.	Glycine	Merck Specialities Pvt. Ltd.	10420105001730
9.	Gum Arabic	S D Fine-Chem Ltd.	I12A/0912/2108/13
10.	Magnesium chloride hexahydrate	Sigma-Aldrich	M7304
11.	Mercuric chloride	Sigma-Aldrich	203777
12.	Olive oil	Figaro	-
13.	Potassium chloride	Merck Specialities Pvt. Ltd.	61779205001730
14.	Sodium dihydrogen phosphate monohydrate	Merck Specialities Pvt. Ltd.	61787405001730
15.	Sodium taurocholate	NZP	-
16.	Sodium hydroxide	Merck Specialities Pvt. Ltd.	61843805001730
17.	Sodium chloride	Merck Specialities Pvt. Ltd.	60640405001730
18.	Trisodium citrate dihydrate	Merck Specialities Pvt. Ltd.	61770905001730
19.	Zinc sulphate heptahydrate	Merck Specialities Pvt. Ltd.	61753005001730

**Appendix 5: List of Instruments**

<b>Sr. No.</b>	<b>Name of the Instrument</b>	<b>Source</b>	<b>Instrument No.</b>
1.	Water bath	Neolab Neolab EXPO HI-TECH Neolab Neolab Neolab Neolab	WRC/ANA-010 WRC/ANA-011 WRC/ANA-012 WRC/PRO-018 WRC/PRO-023 WRC/PRO-024 WRC/PRO-040
2.	Magnetic stirrer	Galaxy	WRC/ANA-006
3.	pH meter	LABINDIA	WRC/ANA-038
4.	Balance (CX 220)	Citizen	WRC/ANA-002
5.	Vortex (CM101)	Remi	WRC-ANA-036
6.	Autotitrator (AT-38C)	Spectralab	WRC/ANA-042
7.	Mixer-grinder	Osterizer	-
8.	Centrifuge C-30.	Remi	WRC/PRO-011



## **Appendix 6: Procedure for the Determination of Optimum pH**

1. **Aim:** To determine the optimum pH of the enzyme.
2. **Scope:** This method is applicable to the pH range from pH 5.0 to pH 10.0.
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:

##### 4.1.1.1. Gum Arabic Solution:

Dissolve 110 g of gum arabic and 12.5 g of calcium chloride dihydrate in a final volume of 1000 ml with distilled water. Stir the solution for about 30 minutes at room temperature to dissolve completely. Centrifuge the solution at 5000 rpm for 20 minutes or filter through a Buchner using Celite as a filter aid. This supernatant/filtrate can be stored at 4°C.

##### 4.1.1.2. Substrate Emulsion:

Place 200 ml of gum arabic solution in a mixer and add 65 ml of olive oil into it. Cool the mixture and emulsify the mixture at high speed for a total of 30 minutes, keeping the temperature below 30°C by repeatedly mixing for 5 minutes and turning the blender off for 1 minute.

##### 4.1.2. 0.5 % (w/v) Sodium taurocholate solution.

##### 4.1.3. 10 mM Sodium Phosphate Buffer, pH 7.0 Containing 1% Sodium Chloride: Prepare 10 mM sodium phosphate buffer, pH 7.0 such that it contains 1 % (w/v) sodium chloride.

##### 4.1.4. 0.02 N Sodium hydroxide solution.

##### 4.1.5. 1% (w/v) Sodium chloride solution.

##### 4.1.6. Enzyme/Sample Preparation:

For the 'Test': Prepare the dilutions in 10 mM sodium phosphate buffer, pH 7.0 containing 1% sodium chloride to obtain the readings within range.

For the 'Blank': Inactivate the enzyme solution prepared for the test by heating in the boiling water bath for 5 -10 minutes.

#### 4.2. Assay Procedure:

- i. Mix 11.5 ml substrate emulsion, 4.5 ml distilled water, 1.0 ml of 0.5% sodium taurocholate solution and 1.9 ml of 1 % sodium chloride solution, equilibrate the mixture at 37°C, and adjust the pH to the desired pH (5.0 - 10.0) using 0.02 N sodium hydroxide.
- ii. Add 0.1 ml of the enzyme sample, and maintain the pH of the reaction for 10 minutes, using 0.02 N sodium hydroxide solution.
- iii. Record the volume of sodium hydroxide consumed for the Test (sample) as 'T<sub>s</sub>' and for the blank as 'T<sub>b</sub>'.

### 5. Calculations:

Unit Definition: One unit of enzyme activity (FIP Unit) is defined as that quantity of a standard lipase preparation (Fungi Lipase International FIP standard) that liberates the equivalent of 1 μmol of fatty acid per minute from the substrate emulsion under the described assay conditions.

**Note**: 1 ml 0.02 N sodium hydroxide corresponds with the neutralization of 20 μmoles of fatty acids.

$$\text{Activity in FIP U/g} = \frac{(T_s - T_b) \times 20}{10 \times 2 \times W}$$

Where,

T<sub>s</sub> = Titre sample.

T<sub>b</sub> = Titre blank.

20 = micromoles of fatty acids liberated by 1 ml of 0.02 N NaOH.

10 = Reaction time in minutes.

2 = Volume of the enzyme in ml.

W = Weight of the enzyme sample in g/ml.

**6. Data Interpretation:**

The pH at which the enzyme shows maximum activity is assigned 100 % activity. The relative activity for each pH is calculated by comparing it to the maximum activity.

## **Appendix 7: Procedure for the Determination of Enzyme Stability at Different pH**

**1. Aim:** To study the effect of pH on the enzyme stability at 25°C.

**2. Scope:** This method is applicable to the pH range from pH 3.0 to pH 10.0.

### **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 % (w/v) Sodium taurocholate solution.

4.1.3. 20 mM Sodium Phosphate Buffer, pH 7.0 Containing 1% Sodium Chloride: Prepare 20 mM sodium phosphate buffer, pH 7.0 using sodium dihydrogen phosphate monohydrate and disodium hydrogen phosphate dodecahydrate such that it contains 1% (w/v) sodium chloride.

4.1.4. 10 mM Sodium Phosphate Buffer, pH 7.0 Containing 1% Sodium Chloride: Prepare 10 mM sodium phosphate buffer, pH 7.0 such that it contains 1 % sodium chloride.

4.1.5. 20 mM Buffer Solutions (pH 3.0 to 10) Containing 1 % Sodium Chloride: Prepare 20 mM buffers, citrate buffer (pH 3.0-5.0), phosphate buffer (pH 6.0 – 8.0) and glycine-NaOH buffer (pH 9.0 – 10.0) such that it contains 1 % sodium chloride.

4.1.6. 0.02 N Sodium hydroxide solution.

4.1.7. 1% (w/v) Sodium chloride solution.

#### 4.1.8. Enzyme/Sample Preparation:

4.1.8.1. pH Stability: Prepare initial dilution in 10 mM sodium phosphate buffer, pH 7.0 containing 1.0% sodium chloride and final dilution in buffer (20 mM) at different pH

(3.0 to 10.0), containing 1.0% sodium chloride, to obtain the readings within range. Incubate the enzyme samples at 25°C for 2 hours.

At the time point, withdraw a sample, dilute with 1.0% sodium chloride solution (to obtain the readings within range), and analyse for activity.

4.1.8.2. Initial Activity: Prepare initial dilution in 10 mM sodium phosphate buffer, pH 7.0 containing 1% sodium chloride and final dilution in sodium phosphate buffer (20 mM), pH 7.0 containing 1% sodium chloride. Dilute the enzyme with 1.0% sodium chloride solution (to obtain readings within range and analyse for activity).

#### 4.2. Assay Procedure:

- i. Mix 11.5 ml substrate emulsion, 4.5 ml distilled water, 1 ml 0.5 % sodium taurocholate and 1.9 ml 1 % sodium chloride solution, equilibrate the mixture at 37°C, and adjust the pH to 7.0 with 0.02 N sodium hydroxide solution.
- ii. For the 'Test', add 0.1 ml of the enzyme sample and maintain the pH at 7.0 for 10 minutes using 0.02 N sodium hydroxide.
- iii. After 10 minutes, abruptly bring the pH to 9.0 and record the volume of sodium hydroxide consumed as 'T<sub>s</sub>'.
- iv. Analyze the 'Blank' in the same manner, except after adjusting the pH to 9.0, add the enzyme and bring the pH back to 9.0. Record the volume of sodium hydroxide consumed as 'T<sub>b</sub>'.

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

#### **6. Data Interpretation:**

The initial enzyme activity obtained as per the standard assay conditions is considered as 100% activity. The percentage residual activity is calculated based on the initial activity.

## **Appendix 8: Procedure for the Determination of Optimum Temperature**

**1. Aim:** To determine the effect of temperature on the enzyme activity.

**2. Scope:** This method is applicable to the temperature range from 25°C to 60°C.

### **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 % (w/v) Sodium taurocholate solution.

4.1.3. 10 mM Sodium Phosphate Buffer Containing 1% (w/v) Sodium Chloride, pH 7.0: Refer to Appendix 7.

4.1.4. 0.02 N Sodium hydroxide solution.

4.1.5. 1 % (w/v) Sodium chloride solution.

4.1.6. Enzyme/Sample Preparation: Prepare the dilutions in 10 mM sodium phosphate buffer, pH 7.0 containing 1% sodium chloride to obtain the readings within range.

#### 4.2. Assay Procedure:

- i. Mix 11.5 ml substrate emulsion, 4.5 ml distilled water, 1 ml 0.5% sodium taurocholate solution and 1.9 ml of 1% sodium chloride solution, equilibrate the mixture at temperatures ranging from 25°C to 60°C, and adjust the pH to 7.0 using 0.02 N sodium hydroxide solution.
- ii. For the 'Test', add 0.1 ml enzyme solution. Maintain the pH for 5 minutes and then abruptly bring the pH to 9.0. Record the volume of sodium hydroxide consumed as 'T<sub>s</sub>'.
- iii. Analyze the 'Blank' in the same manner, except after equilibration bring the pH to 9.0, add the enzyme sample and again bring the pH back to 9.0.

- iv. Record the volume of sodium hydroxide consumed as  $T_b$ .

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

**6. Data Interpretation:**

The temperature at which the enzyme shows maximum activity is assigned 100 % activity. The relative activity for each temperature is calculated by comparing it to the maximum activity.

## **Appendix 9: Procedure for the Determination of the Enzyme Stability at Different Temperatures**

**1. Aim:** To determine the effect of temperature on the enzyme stability.

**2. Scope:** This method is applicable to the temperature range from 25°C to 55°C.

### **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 % (w/v) Sodium taurocholate solution.

4.1.3. 10 mM Sodium Phosphate Buffer Containing 1% Sodium Chloride, pH 7: Refer to Appendix 7.

4.1.4. 0.02 N Sodium hydroxide solution.

4.1.5. 1% (w/v) Sodium chloride solution.

4.1.6. Enzyme/Sample Preparation: Prepare the dilutions in 10 mM sodium phosphate buffer, pH 7.0 (containing 1% sodium chloride) to obtain readings within range.

For the Stability: Incubate the enzyme sample at temperatures ranging from 25 - 55°C for 2 hours.

For Initial Activity: Do not incubate the enzyme sample at any temperature.

#### 4.2. Assay Procedure:

- i. Mix 11.5 ml substrate emulsion, 4.5 ml distilled water, 1.0 ml 0.5% sodium taurocholate solution and 1.9 ml 1 % sodium chloride solution, equilibrate the mixture at 37°C, and adjust the pH of the solution to 7.0 using 0.02 N sodium hydroxide.
- ii. For the Test, add 0.1 ml of the enzyme sample.
- iii. Maintain the pH of the reaction at 7.0 for 10 minutes, and then abruptly bring the pH to 9.0. Record the volume of sodium hydroxide consumed as  $T_s$ .



- iv. Analyze the blank in the same manner, except after equilibration bring the pH to 9.0, add the enzyme and again bring the pH to 9.0. Record the volume of sodium hydroxide consumed as  $T_b$ .

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

**6. Data Interpretation:**

The initial enzyme activity obtained as per the standard assay conditions is considered as 100% activity. The percentage residual activity is calculated based on the initial activity.