Soni & Associates Inc.

749 46th Square
Vero Beach, FL 32968, USA
Telephone: 772-299-0746
Facsimile: 772-299-5381
E-mail: sonim@bellsouth.net

June 30, 2022

Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety (HFS-200)
5100 Campus Drive
College Park, MD 20740

Subject: GRAS Notification for the intended use of Maltogenic amylase from *Geobacillus stearothermophilus* produced by *Escherichia coli* BLASC (SD-6849)

Dear Sir/Madam:

Pursuant to 21 CFR Part 170, Subpart E, Advanced Enzymes Technologies, Ltd., through me as its agent, hereby provide notice of a claim that the intended use of Maltogenic amylase from *Geobacillus stearothermophilus* produced by *Escherichia coli* BLASC (SD-6849) in baking, brewing, and starch processing is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because Advantage Enzymes Technologies has determined that the intended use is generally recognized as safe (GRAS) based on scientific procedures.

As required, please find enclosed three copies of the notification. If you have any questions or require additional information, please feel free to contact me by phone at +1-772-299-0746 or by email at sonim@bellsouth.net.

Sincerely,

Madhu G. Soni, PhD, FACN, FATS

Agent for
Advanced Enzymes Technologies, Ltd.,
INDIA

Enclosure: Three copies of GRAS notification

GRAS NOTIFICATION

Maltogenic amylase from Geobacillus stearothermophilus produced by Escherichia coli BLASC (SD-6849)

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

Table of Contents

List of Tables

List of figures

List of Abbreviations

%	Percentage
µg	Microgram
ADI	Acceptable Daily Intake
AOAC	Association of Official Agricultural Chemists
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
bp	Base Pairs
BP	British Pharmacopoeia
BSL-1	Biosafety Level 1
bw	Body Weight
°C	Degrees Celsius
CFU	Colony Forming Unit
cGMP	Current Good Manufacturing Practice
CLSI	Clinical and Laboratory Standards Institute
d	Day
DBETH	Database of Bacterial ExoToxins for Human
DE	Dextrose Equivalent
DNA	Deoxyribonucleic Acid
DP	Degrees of Polymerization
E. coli	*Escherichia coli*
E.C. number	Enzyme Commission Number
EC regulation	European Council Regulation
EDI	Estimated Daily Intake
EFSA	European Food Safety Authority
EP (Ph. Eur.)	European Pharmacopoeia
ETA	Enzyme Technical Association
FALCPA	Food Allergen Labelling and Consumer Protection Act
FAO	Food and Agriculture Organization
FARRP	Food Allergy Research and Resource Program
FASTA	FAST-All
FDA	U.S. Food and Drug Administration
FF	Final Food
FNP	Food and Nutrition Paper
FSANZ	Food Standards Australia New Zealand
g	Gram
GLP	Good Laboratory Practice
GRAS	Generally Recognized As Safe
GRN	GRAS Notice
h	Hour

HACCP	Hazard Analysis and Critical Control Points
IL	Interleukin
IP	Indian Pharmacopoeia
IPTG	Isopropyl-β-D-thiogalactopyranoside
ISO	International Organization for Standardization
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kDa	Kilodalton
kg	Kilogram
L	Litre
LD_{50}	Median Lethal Dose
MANU	Maltogenic Amylase Unit
MCS	Multiple Cloning site
mg	Milligram
ml	Millilitre
MoS	Margin of Safety
n	Number
NHC	National Health Commission
NLT	Not Less Than
NMT	Not More Than
NNS	National Nutrition Survey
NOAEL	No Observed Adverse Effect Level
OECD	Organization for Economic Co-operation and Development
PCR	Polymerase Chain Reaction
PID	Percentage Identity
QPS	Qualified Presumption of Safety
RAPD	Random Amplified Polymorphic DNA
RM	Raw Material
RNA	Ribonucleic Acid
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
SGF	Simulated Gastric Fluid
Spp.	Species Pluralis (multiple species)
TMDI	Theoretical Maximum Daily Intake
TOS	Total Organic Solid
USC	United States Code
USDA	United States Department of Agriculture
USP	United States Pharmacopeia
WHO	World Health Organization
µmol	Micromole

Part 1. 21 CFR 170.225: Signed Statements and Certifications

1.1 GRAS Notice Submission

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

1.2 Name and Address of Notifier

APPLICANT

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

PERSON RESPONSIBLE FOR THE DOSSIER

Name:	Ankit Kishor Rathi, Sr. Manager – Regulatory Affairs Advanced Enzyme Technologies Ltd.
Address:	5th Floor, 'A' wing, Sun Magnetica LIC Service Road, Louiswadi
Postal code and City:	Thane (W), India 400604
Country:	India
Tel. no:	+91 22 25830284
E-mail:	ankit.rathi@advancedenzymes.com

AGENT WHO IS AUTHORIZED TO ACT ON BEHALF OF THE NOTIFIER

Name:	Madhu Soni. *PhD, FACN, FATS* Soni & Associates Inc.
Address:	749 46th Square
Postal code and City:	Vero Beach, FL 32968
Country:	United States of America
Tel. no:	772-299-0746
E-mail:	sonim@bellsouth.net

1.3 Name of Notified Substance

The notified substance is 'Maltogenic amylase' from *Geobacillus stearothermophilus* produced by genetically modified *Escherichia coli* BLASC.

'BLASC' is the designation of the *E. coli* strain used for the production of maltogenic amylase by Advanced Enzyme Technologies Ltd. The strain is deposited with the American Type Culture Collection (ATCC), United States of America (USA) under strain designation SD-6849.

Maltogenic amylase (EC: 3.2.1.133, CAS no.: 160611-47-2, Systematic name: 4-α-D-glucan α-maltohydrolase) is glucan maltohydrolase catalyses the hydrolysis (1→4)-alpha-D-glucosidic linkages in polysaccharides.

The product maltogenic amylase from *E. coli* BLASC (SD-6849) is a powder preparation. Commercial preparations are known as SEBake Fresh 1.5P, SEBake Fresh 10P, SEBake Fresh 20P, SEBake Fresh 50P, SEBake Fresh100P.

In this GRAS notice, Maltogenic amylase from *E. coli* BLASC is referred to by names such as 'Maltogenic amylase', 'Maltogenic alpha amylase', 'Maltogenic amylase from *E. coli* (strain BLASC)', 'Maltogenic amylase from *Geobacillus stearothermophilus* produced by genetically modified *Escherichia coli* (strain BLASC)'

1.4 Intended Conditions of Use

Maltogenic amylase from *E. coli* BLASC is intended to be used in baking, brewing, and starch processing.

The enzyme maltogenic amylase (E.C. 3.2.1.133) is intended to be used as a processing aid during food manufacturing and it does not have a technical role in the final food. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements under current Good Manufacturing Practices. The "general" population is the target population for consumption.

1.5 Statutory Basis for GRAS Status

Advanced Enzyme Technologies Ltd., has determined that the intended use of maltogenic amylase from *E. coli* BLASC is GRAS through scientific procedures in accordance with 21 CFR §170.30(a) and (b).

1.6 Premarket Exempt Status

Since Advanced Enzyme Technologies Ltd. has determined that the intended use of maltogenic amylase from *E. coli* BLASC is GRAS, the use of the notified substance is exempt from pre-market approval requirements of the Federal Food, Drug, and Cosmetic Act.

1.7 Data Availability

Advanced Enzyme Technologies Ltd. agrees to make the data and information that are the basis for the evaluation of GRAS status available to FDA upon request. Such data and information may be sent by Advanced Enzyme Technologies Ltd. to FDA either in electronic format or on paper or reviewed during customary business hours at the above mentioned address.

1.8 FOIA Statement

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

1.9 Certification

To the best of my knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to me and pertinent to the evaluation of the safety and GRAS status of the intended use of maltogenic amylase from *E. coli* BLASC.

1.10 FSIS Statement

Not applicable.

1.11 Signature of Responsible Party or Agent

Madhu Soni, *PhD, FACN, FATS*
Soni & Associates Inc.

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

2.1 Identity/ Identification

The subject of this GRAS notification is a maltogenic alpha-amylase produced by submerged fermentation of a genetically modified *Escherichia coli* (strain BLASC) microorganism expressing the gene encoding for a maltogenic alpha-amylase from *Geobacillus stearothermophilus*.

2.1.1 *SCIENTIFIC NAME, TAXONOMY AND OTHER NAMES*

Name of the enzyme protein:	Maltogenic amylase
Synonyms:	1,4-α-D-glucan α-maltohydrolase
CAS no.:	160611-47-2
EC (IUBMB) number:	EC 3.2.1.133

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

EC 3	Hydrolases
EC 3.2	Glycosylases
EC 3.2.1	Glycosidases, i.e. enzymes hydrolyzing O- and S-glycosyl compound
EC 3.2.1.133	Glucan 1,4-alpha-maltohydrolase

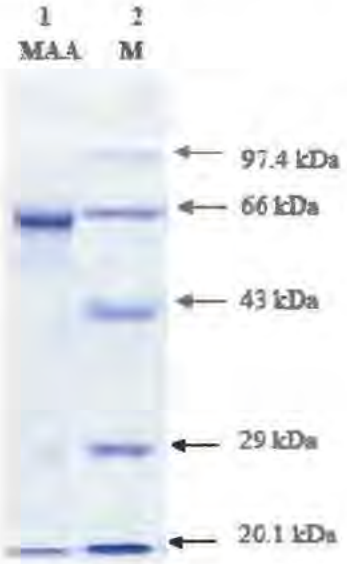
2.1.2 *AMINO ACID SEQUENCE AND MOLECULAR WEIGHT OF ENZYME PROTEIN*

Maltogenic amylase from *E. coli* BLASC is comprised of 686 amino acids (calculated molecular weight of 75.2 kDa) having following sequence:

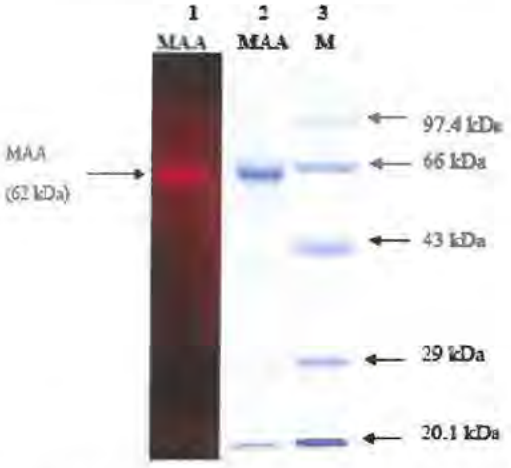
SSSASVKGDVIYQIIIDRFYDGDTTNNNPAKSYGLYDPTKSKWKMYWGGDLEGVRQK
LPYLKQLGVTTIWLSPVLDNLDTLAGTDNTGYHGYWTRDFKQIEEHFGNWTTFDTLV
NDAHQNGIKVIVDFVPNHSTPFKANDSTFAEGGALYNNGTYMGNYFDDATKGYFHH
NGDISNWDDRYEAQWKNFTDPAGFSLADLSQENGTIAQYLTDAAVQLVAHGADGLR
IDAVKHFNSGFSKSLADKLYQKKDIFLVGEWYGDDPGTANHLEKVRYANNSGVNVL
DFDLNTVIRNVFGTFTQTMYDLNNMVNQTGNEYKYKENLITFIDNHDMSRFLSVNSN
KANLHQALAFILTSRGTPSIYYGTEQYMAGGNDPYNRGMMPAFDTTTTAFKEVSTLA
GLRRNNAAIQYGTTTQRWINNDVYIYERKFFNDVVLVAINRNTQSSYSISGLQTALPN
GSYADYLSGLLGGNGISVSNGSVASFTLAPGAVSVWQYSTSASAPQIGSVAPNMGIPG
NVVTIDGKGFGTTQGTVTFGGVTATVKSWTSNRIEVYVPNMAAGLTDVKVTAGGVS
SNLYSYNILSGTQTSVVFTVKSAPPTNLGDKIYLTGNIPELGNWSTDTSGAVNNAQGP
LLAPNYPDWFYVFSVPAGKTIQFKFFIKRADGTIQWENGSNHVATTPTGATGNITVTW
QN

Molecular weight of the maltogenic amylase from *E. coli* BLASC was determined as 62 kDA following SDS-PAGE, and with gel permeation chromatography method, indicating that the enzyme is a monomer.

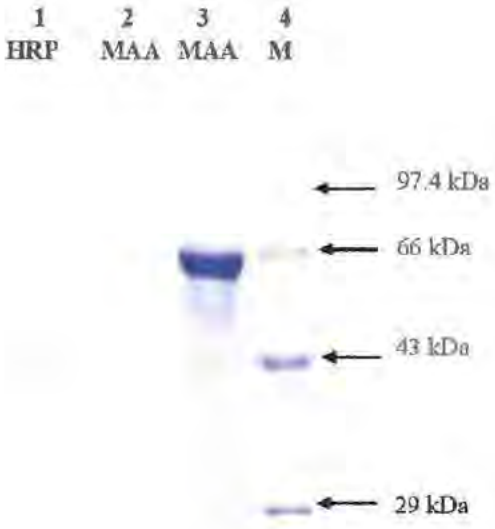
The maltogenic amylase sample was analyzed on a 10% SDS-PAGE gel. Our zymogram analysis indicated that the protein at 62 kDa, has amylase activity. Schiff's staining of the SDS-PAGE gel showed that this protein is not glycosylated. SDS PAGE analysis of maltogenic amylase derived from *E. coli* BLASC. (Refer to Fig. 1, 2 & 3)



Lane 1- MAA: Maltogenic alpha amylase Lane 2- M: Molecular Weight Marker
Fig -1 Electrophoresis of maltogenic amylase preparation from *E. coli* BLASC



Lane 1: Zymogram-Lane 1: MAA-Maltogenic alpha amylase
Lane 2-3: Coomassie Staining- Lane 2: MAA-Maltogenic alpha amylase Lane 3: M-Protein markers
Fig- 2 Zymogram of maltogenic amylase



Lane 1-2: Schiff's Staining: Lane 1: Horse radish peroxidase (HRP), positive control
Lane 2: MAA-maltogenic alpha amylase
Lane 3-4: Coomassie Staining: Lane 3: MAA-maltogenic alpha amylase
Lane 4: M-Protein markers

Fig-3 Schiff's staining of a maltogenic amylase

2.1.3 *ENZYME ACTIVITY*

Maltogenic amylase catalyzes the hydrolysis of (1→4)-alpha-D-glucosidic linkages in starch polysaccharides, to successively remove maltose from the non-reducing chain ends.

The enzyme assay method was based on monograph prepared by JECFA (1999) and Korea Food Additives Code. The enzyme activity was measured spectrophotometrically using maltotriose as the substrate. One unit of enzyme activity is defined as the amount of enzyme that cleaves 1 μmol of maltotriose per minute under the given assay conditions.

To the best of our knowledge, the maltogenic amylase from *E. coli* BLASC described in this dossier does not possess any detectable 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, maltogenic amylase from *E. coli* BLASC was measured under various pH and temperature conditions.

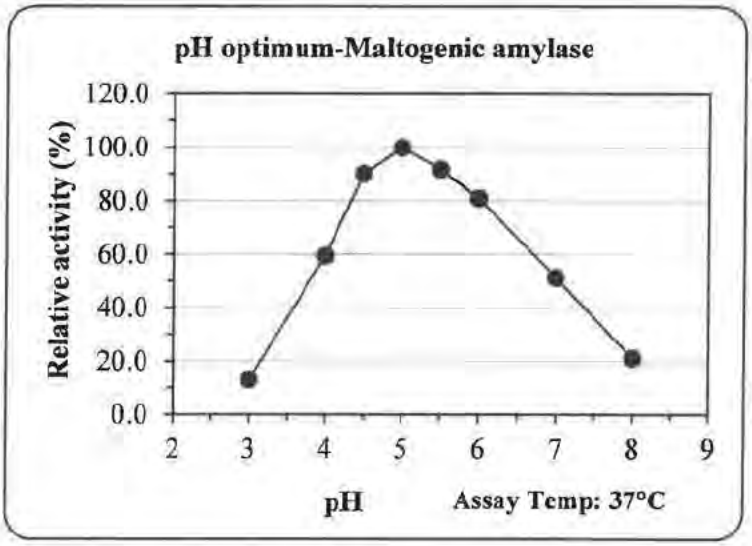


Fig.4: pH optimum of the maltogenic amylase

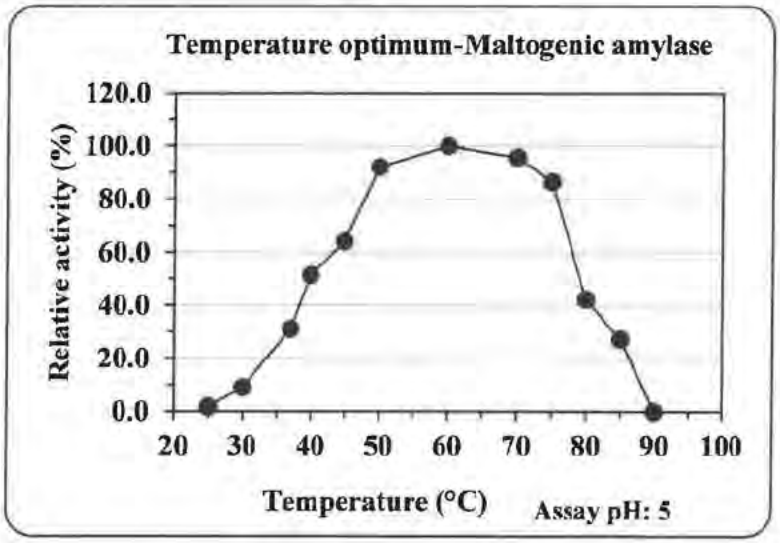


Fig5: Temperature optimum of the maltogenic amylase

The maltogenic amylase exhibited activity between pH 3.0 to 8.0 (Fig. 4) and temperature 25°C to 90°C, with pH 5.0 and temperature 60°C as optimum for activity (Fig. 5).

2.1.3.2 Information on the stability of the food enzyme

The stability of the food enzyme, maltogenic amylase from *E. coli* BLASC was measured under various pH and temperature conditions.

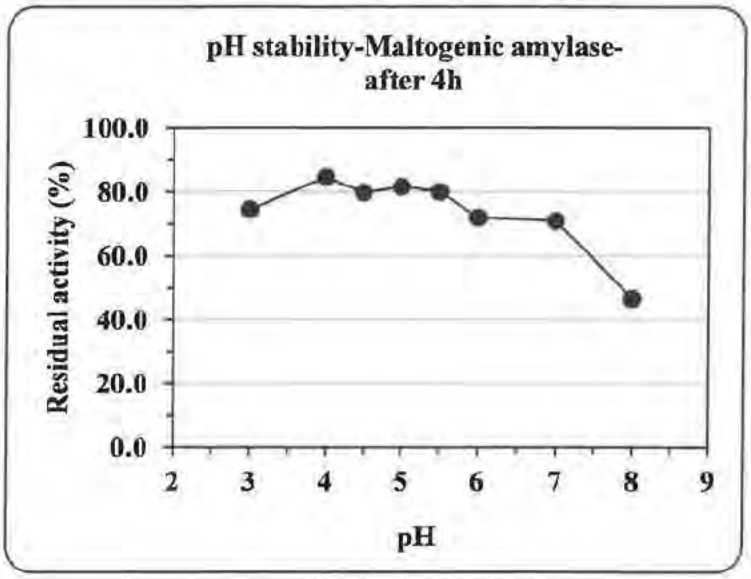


Fig.6: pH stability of the maltogenic amylase

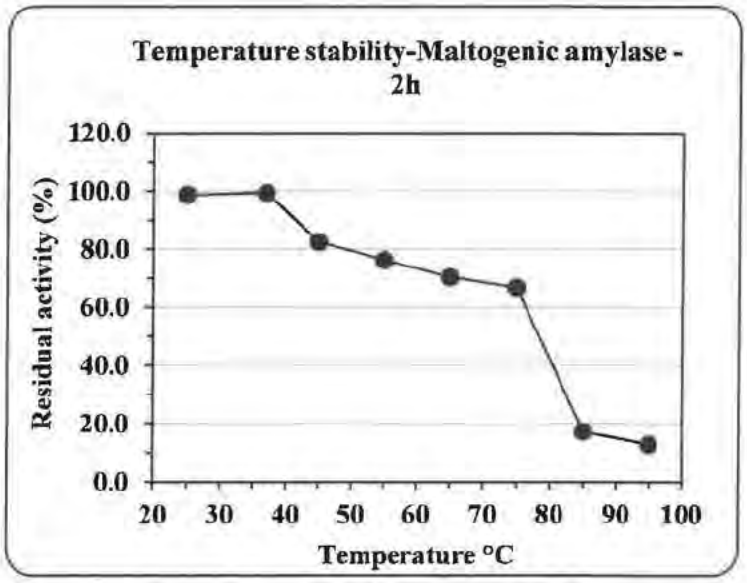


Fig.7: Temperature stability of the maltogenic amylase

The maltogenic amylase from *E. coli* BLASC was stable between pH 4.0 to 5.5 for 4 hours (Fig. 6). The enzyme was found to be stable between 25 to 65°C for 2 hours. The enzyme activity decreases rapidly at temperatures higher than 75°C (Fig. 7).

2.2 Production organism

2.2.1 *SCIENTIFIC NAME, TAXONOMY AND OTHER NAMES*

The microorganism used for the production of the maltogenic amylase is a genetically modified *Escherichia coli* strain BLASC.

The strain is described as *E. coli* BLASC throughout the dossier.

The production strain *E. coli* BLASC was identified following 16S rRNA gene as phylogenetic marker using primers 16SF27 and 16SR1525. The BLAST and phylogenetic analysis of 16S rRNA gene sequence showed 100% homology to *Escherichia coli* BL21 (DE3).

Taxonomy:

Name	:	*Escherichia coli* BLASC
Kingdom	:	Bacteria
Phylum	:	Proteobacteria
Class	:	Gamma Proteobacteria
Order	:	Enterobacteriales
Family	:	Enterobacteriaceae
Genus	:	*Escherichia*
Species	:	*Coli*
Generic Name	:	*Escherichia coli* BLASC
Commercial Name	:	*Escherichia coli* BLASC

The name of the source organism in the dossier is mentioned as *Escherichia coli* BLASC. The strain designation *Escherichia coli* BLASC and *E. coli* BLASC are used interchangeably while referring to the production strain, in certain sections of the dossier.

2.2.2 *PRODUCTION STRAIN, RECIPIENT AND DONOR, STRAIN*

Production strain:

The recombinant production strain *E. coli* BLASC was identified as *Escherichia coli* BL21 (DE3) using 16S rRNA gene as a phylogenetic marker. The production strain *E. coli* BLASC is a non-pathogenic, non-toxigenic strain generated using recombinant DNA technology with the host strain *E. coli* SAML27 [derivative of *E. coli* BL21(DE3)] based on post segregational killing system. Post-segregational killing system is comprised of genes *ccd*B and *ccd*A, which are isolated from F plasmid of *E. coli*. In the host *E. coli* SAML 27, the gene *ccd*B is integrated with the host genome, while the gene *ccd*A is present on the plasmid along with *amy*M gene. The segregation (loss) of the plasmid from the cells results in cell death due to the expression of *ccd*B gene, which is otherwise ineffective due to the presence of *ccd*A. This system is therefore named as 'post-segregational killing' system. It ensures the growth of only plasmid harboring cells. The maltogenic amylase expressing plasmid (pTA-amyM) containing

the DNA sequence of the maltogenic amylase gene with the regulatory elements and *ccd*A gene was transformed to the recipient strain *E. coli* SAML27 to generate *E. coli* BLASC

The target of the post-segregational killing system is the bacterial DNA gyrase not present in eukaryotic cells and consequently, has no action on these cells.

Recipient strain:

The recipient strain *E. coli* SAML27 is a derivative of *E. coli* BL21 (DE3) an established non-pathogenic laboratory strain used for the production of various recombinant proteins (Rosano et al., 2014; Gopal et al., 2013; Huang et al., 2012; Liu et al., 2015, Jia et al., 2016). The recipient strain contained *ccd*B gene of the post segregational killing system, the effect of which would be negated by *ccd*A expressed in the maltogenic amylase plasmid (pTA-amyM), eventually supporting the growth of only those cells which contain the plasmid.

The safety of *E. coli* BL21 has been established by different authors through bioinformatics (whole genome sequence) and experimental evidence. Jeong et al. (2009), through whole genome, demonstrated that *E. coli* BL21 does not contain functional/intact gene sequences encoding an O antigen polysaccharide; it also lacks the well-recognized pathogenic mechanisms common in *E.coli* strains causing the majority of enteric infections. Studier et al. (2009) confirmed the absence of *E. coli* enterotoxins or related pathogenic determinants through whole genome analysis of *E. coli* BL21 (DE3), which is consistent with the long history of safe use of these strains in the laboratory and in the production of food additives and pharmaceutical preparations. Experimentally, the absence of toxin production by *E. coli* BL21 was demonstrated in the feeding studies, where oral and/or intraperitoneal administration of *E. coli* BL21 to mice, 1-day-old chicks, and sheep were without evidence of intolerance or toxicity (Chart et al., 2000). The absence of toxin genes renders this host suitable for the production of therapeutic proteins (Maksum et al., 2020). Short-term and sub-chronic toxicity evaluations of recombinant proteins produced by *E. coli* BL21 (DE3) showed no evidence of toxicity from the organism during fermentation (Mathesius et al., 2009; Guimarães et al., 2010; Quemada et al., 2010).

The evidence, described above, render safe use of *E. coli* BL21 (DE3) strains for production of recombinant proteins and support the conclusion that *E. coli* BL21 (DE3) is a non-toxigenic strain.

Donor strain:

The maltogenic amylase donor microorganism - *Geobacillus stearothermophilus* - is a member of *Bacillus* species group, which has a long history of safe use in industrial-scale enzyme production and is considered safe for production of enzymes for food as well as feed processing and numerous other industrial applications. Importantly, EFSA has assigned *Geobacillus stearothermophilus* a status of "Qualified Presumption of Safety" (QPS) (EFSA 2007, 2020a). Expressing maltogenic amylase gene *amy*M from this organism, therefore, doesn't cause a safety concern.

2.2.3 *GENETIC MODIFICATION*

The generation of the recombinant production strain *E. coli* BLASC involved 3 different stages:

1. Generation of host strain *E. coli* SAML27 by integration of *ccd*B gene of the post segregational killing system in *E. coli* BL21(DE3)
2. Generation of plasmid pTA-amyM containing the maltogenic amylase gene with regulatory elements and the *ccd*A gene responsible for post segregational killing. The vector pTA is a plasmid with *ccd*A gene of post-segregational killing system, pBR322 and f (+) origin of replication. pTA was utilized for cloning of *amy*M gene to generate pTA-amyM for expression of maltogenic amylase in *E. coli* SAML27
3. Transformation of the plasmid pTA-amyM to *E. coli* SAML27 to generate recombinant production strain *E. coli* BLASC producing maltogenic amylase enzyme.

The post-segregational killing technology used to generate the production strain *E.coli* BLASC, ensures stabilization of the plasmid in cells during cell growth, throughout the fermentation process. Only the cells carrying the plasmid can grow, as the loss of plasmid results in cell death.

The *ccd*B and *ccd*A genes were PCR amplified from the F plasmid of *E. coli.* The genetic modification was performed on *E. coli* BL21 (DE3) by integrating the *ccd*B gene through P1 transduction. The selection of the desired clones was based on an antibiotic resistance gene, which was later removed using flippase (flp) site-specific recombinase acting on the flippase recognition target (FRT) sites flanking the antibiotic resistance gene resulting in the final recipient strain *E. coli* SAML27. The removal of antibiotic resistance gene was verified phenotypically, using PCR technique and by sequencing the recombination region.

The *ccd*A gene of post-segregational killing system under the control of pmob promoter was cloned into the pET21 plasmid. The ampicillin gene on the plasmid was removed by restriction digestion and self-ligation to generate the plasmid pTA. The synthetic gene *amy*M (2064 bp) was cloned to pTA (devoid of antibiotic marker) to generate pTA-amyM (6913 bp). The plasmid pTA-amyM was transformed to *E.coli* SAML27 resulting in the production strain *E.coli* BLASC. The genetic construction was evaluated at every step for antibiotic sensitivity and intended integration in the chromosome by PCR technique and sequencing of the recombination region.

The absence of 3 antimicrobial resistance (AMR) genes, viz ampicillin, kanamycin and chloramphenicol, used during the generation of the production strain was verified by the PCR amplification of the AMR genes, using *E. coli* BLASC and respective controls as template. No PCR product was observed when the production strain *E. coli* BLASC was used as the template. The results indicated no functional antibiotic resistance genes, used during the construction of the production strain, to be present in the final strain generated through genetic

modification. Also, the plasmid pTA-*amy*M was sequenced to confirm the absence of any AMR gene.

2.2.4 *INFORMATION ON GENETIC STABILITY*

The genome of Genetically Modified Microorganism (GMM) should be stable during storage (master cell bank), sub-culture (working cell bank) and production process. The post-segregational killing technology used in the generation of the production strain, ensures stabilization of the plasmid during the fermentation process. Only the cells carrying the plasmid containing the *amy*M gene will grow as the loss of plasmid results in the cell death.

To demonstrate the genetic stability of the production strain *E. coli* BLASC, the stability of the genetic traits in the GMM was demonstrated using the genetic fingerprinting technique, Random Amplified Polymorphic DNA (RAPD). Two primers P2 and 15002 were selected based on the distinct profile for determining the stability of recombinant production strain *E. coli* BLASC during the production of maltogenic amylase. 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 production strain *E. coli* BLASC 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 mentioned above.

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

The source material used for the production of maltogenic amylase covered in this dossier is genetically modified *E. coli* BLASC.

E. coli strains such as *E. coli* K-12, B, C, W and their derivatives are all classified as risk group 1 organisms in biosafety guidelines (Bauer et al., 2007). These strains are frequently used for recombinant protein production and have a history of safe use.

During the recent years, considerable knowledge and experience on the safe use of recombinant *E. coli* strains at industrial scale has been accumulated. *E. coli* K-12 strain can be used under the lowest containment level at large scale, GILSP, as defined by OECD (1992).

E. coli BL21 is a common host microorganism for the expression of genes and known to produce several recombinant biopharmaceuticals and enzymes at industrial scale (Bauer et al., 2007, Kamionka, 2011). Most common therapeutic proteins expressed in *E. coli* are human insulin (Nilsson et al., 1996), interleukin 2 (IL-2) (Roifman et al., 1985), etc. Several industrial enzymes have also been produced in *E. coli* viz. xylanase (Whitehead and Espell, 1989), betaglucosidase (Ferreira et al., 2018), cellulases (Amraini et al., 2017), alpha cyclodextrin glycotransferase, etc. Thus, *E. coli* strains can be considered as a safe production organism for the enzymes used in food/feed processing as well as numerous other industrial applications.

Furthermore, *E. coli* BL21 strain has been used as a laboratory organism for decades without reported incidents of infection. It does not produce toxins that cause illness by ingestion, such as Shiga-like toxin produced by certain toxigenic strains of *E. coli.* (Maksum et al., 2020).

The EFSA has provided positive scientific opinions for the substances produced by the selected strains of *E. coli*. (EFSA Journal 2014b, EFSA Journal 2019)

United States FDA has also issued "no questions" letters in response to GRAS notices for substances produced by the selected strains of *E. coli* and supporting its position as safe for use in food production (GRN 624). The production of rennin by *E. coli* K-12 was affirmed as GRAS by the FDA in 1990.

2.2.6 *ABSENCE OF THE PRODUCTION ORGANISM IN THE PRODUCT*

Absence of production organism (*E. coli* BLASC) in the product was demonstrated following EFSA guidance (EFSA Journal 2011).

Three production batches of the maltogenic amylase enzyme were analysed for the presence/absence of the production microorganism. The production microorganism was not detected in the tested production batches. The positive control, viz the enzyme sample (1 g) spiked with one cell showed growth on the selected medium, indicating the technique is suitable to detect the presence of even 1 cell of the production microorganism in the enzyme sample. The results indicated absence of recombinant production strain *E. coli* BLASC in all the three batches analysed.

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

To demonstrate the absence of transferable recombinant DNA sequences in the product, the following parameters were considered during the method development:

1. Total DNA extraction procedure ensured that all DNA from non-viable cells, potentially remaining in the product, is recovered.
2. The primers designed to amplify a fragment of targeted sequence (recombinant DNA) should cover a maximum 1 kb gene fragment size.
3. Detection limit of recombinant DNA in the enzyme product was determined by spiking the control DNA in different dilutions until DNA extinction.
4. The detection limit of the target DNA in the product was ensured to be 10 ng of DNA per gram of the enzyme product.

Maltogenic amylase produced by the genetically modified *E. coli* BLASC was analysed for the presence of recombinant DNA sequence using PCR based detection method for a gene fraction spanning 942 bp of the PCR product. The DNA extraction method developed was capable of recovering DNA from the high protein containing enzyme product. Also, a robust PCR master mix was utilized for standardization of PCR method which could detect up to picogram level of recombinant DNA.

For analysis of the maltogenic amylase enzyme samples, DNA was extracted from 1 gram of maltogenic amylase enzyme product and also from the enzyme product spiked with known amount of DNA (maltogenic amylase enzyme product positive control). PCR positive and negative controls were included to ensure functional PCR

Three batches of the maltogenic amylase enzyme, produced with *E. coli* BLASC, when analysed in triplicate, did not show detectable amounts of recombinant DNA (The detection limit for less than 1 kb of recombinant DNA for maltogenic amylase gene was 10 ng). This was confirmed due to absence of amplicon (942 bp), when the extracted DNA from the enzyme product was used as template. Expected results were obtained for respective positive and negative controls.

2.2.8 SAFETY ASPECTS OF GENETIC MODIFICATION

Description of genetic trait(s) or phenotypic characteristics and in particular, any new traits and characteristics, which may be expressed or no longer expressed

The plasmid pTA-amyM was transformed to *E. coli* SAML27 bacteria for the expression of *amy*M gene and production of the maltogenic amylase protein.

The production strain *E. coli* BLASC contains the post-segregational killing system, ensuring the growth of only host cells with the plasmid. The two gene components are negatively autoregulated at the level of transcription, probably by a complex comprising the two gene products. The *ccd*B integrated into the genome by itself has no regulatory activity or operator DNA-binding affinity and needs the *ccd*A gene in order to effect transcriptional control. These two genes were amplified by PCR technique from the F plasmid of *E. coli* (non-pathogen). The target of the post-segregational killing system is the bacterial DNA gyrase, which is not present in eukaryotic cells and consequently has no action on these cells. The *ccd*A protein is able to counteract the action of the *ccd*B protein by repressing the expression. Consequently, no expression of any detrimental gene is observed when the cells carry both the genes.

The genetic modifications carried out for the generation of *E. coli* BLASC do not pose any safety concerns.

Structure and amount of any vector and/or donor nucleic acid remaining in GMM

The strategy for generating the recombinant production strain *E. coli* BLASC producing maltogenic amylase involved two gene component system. Gene component 1 (*ccd*B) integrated and retained in the genome of the final strain *E. coli* BLASC for stabilizing the plasmid. The cloning of the maltogenic amylase gene (*amy*M) to the expression vector pTA utilizes the post-segregational killing system to stabilize the plasmid. The vector is completely stable in the production host even without antibiotic resistance marker due to the stabilization system based on post-segregational killing system. The expression of maltogenic amylase is episomal and the introduced DNA remains on the vector transformed to *E. coli* SAML27.

2.2.9 *ABSENCE OF ANTIBIOTIC GENES AND TOXIC COMPOUNDS*

Antibiotic resistance genes, which were used during the generation of the production strain *E. coli* BLASC were not retained in the final strain. The absence of 3 antimicrobial resistance (AMR) genes viz ampicillin, kanamycin and chloramphenicol used during the generation of the production strain was verified phenotypically and by carrying out the PCR amplification of the AMR genes using *E. coli* BLASC and respective controls as template. No PCR product was observed when the production strain *E. coli* BLASC was used as the template. The results indicated no functional antibiotic resistance genes, used during the construction of the production strain, retained into the final strain generated through genetic modification.

The production strain was analyzed for antibiotic susceptibility/resistance following CLSI (2012) and EFSA (2008, 2009) method and found resistant to antibiotics such as fosfomycin and colistin. In fact, the recipient strain *E. coli* SAML27 [derivative of *E. coli* BL21 (DE3)], from which the production strain *E. coli* BLASC is derived, was also found resistant to fosfomycin and colistin, confirming that the resistance to fosfomycin and colistin is inherent in the production host and not acquired in the genetic modification process to express the maltogenic amylase gene

The colistin and fosfomycin resistance mechanism in *E. coli* BL21(DE3) is described in the literature. Xu et al. (2021) in their study on the molecular basis of colistin resistance in *E. coli* BL21(DE3) indicated that the 3'downstream region of *pmrB* is critical for the PmrB-mediated lipid A modifications and colistin resistance in *E. coli* BL21(DE3), suggesting a novel regulatory mechanism of PmrB-mediated colistin resistance in *E. coli.* Based on the literature and the whole genome sequence analysis of *E. coli* BL21(DE3) (https://www.ncbi.nlm.nih.gov/nuccore/CP001509), the production organism *E. coli* BLASC could be resistant to fosfomycin due to the impairment of the transport system.

Bacterial toxins are virulence factors that manipulate host cell functions and take over the control of vital processes of living organisms to favour microbial infection. The production strain *E. coli* BLASC is derived from *E. coli* BL21(DE3), which is known for its safe usage. Experimentally, the absence of toxin production by *E. coli* BL21 was demonstrated in the feeding studies, where oral and/or intraperitoneal administration of *E. coli* BL21 to mice, 1-day-old chicks, and sheep were without evidence of intolerance or toxicity (Chart et al., 2000). Short-term and sub-chronic toxicity evaluations of recombinant proteins produced by *E. coli* BL21 (DE3) showed no evidence of toxicity (Mathesius et al., 2009; Guimarães et al., 2010; Quemada et al., 2010).

To confirm that the maltogenic amylase from *E. coli* BLASC is non-toxic, a sequence homology assessment of the maltogenic amylase enzyme with known toxins was conducted. The amino acid sequence was aligned with the known toxin sequences available in the "Database of Bacterial ExoToxins for Human (DBETH)". It consists of structures, interaction networks and analytical results for 229 exotoxins, from 26 different humans' pathogenic bacterial genus. All toxins in the database are classified into 24 different toxin classes. The

amino acid sequence of the maltogenic amylase provided in section 2.1.2 was used as input for the search.

Maltogenic amylase protein did not show homology with known toxin protein sequences described in database sequences.

2.2.10 SECONDARY METABOLITES

Most industrial strains of *E coli* 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 6.5 for details)

The production strain, *E. coli* BLASC does not produce secondary metabolites of toxicological concern to the human. The strain *E. coli* BLASC was evaluated for its antimicrobial activity following CLSI (2012) guidelines and EFSA (2008 & 2009) and found not to produce antimicrobials (Refer to section 6.2.2 for details). The safety of the production organism was further substantiated by lack of toxicity when the maltogenic amylase from *E. coli* BLASC was tested in standard *in vitro* and *in vivo* toxicity model systems (Refer to section 6.4 for details).

2.3 Manufacturing process

2.3.1 *OVERVIEW*

Maltogenic amylase is produced by a fed-batch fermentation process. Fermentation is carried out following current Good Manufacturing Practice (cGMP) and the principles of Hazard Analysis and Critical Control Points (HACCP). The manufacturing facility is ISO 9001, ISO 22000 (Food Safety Management System) and GMP certified in accordance with 21CFR§110 and 21CFR§117.

Maltogenic amylase is produced by fermentation. Fermentation is a well-known process that occurs in food and has been used for the production of food enzymes for decades. Liquid state or submerged fermentation is used to produce the maltogenic amylase.

As shown below, the key steps for the production of the maltogenic amylase are fermentation, recovery, formulation, and packaging. The process is illustrated in Fig 8.

2.3.2 *FERMENTATION*

2.3.2.1 Raw materials

Materials used in the fermentation and post-fermentation processes (inoculum, seed, main fermentation, recovery, and formulation) are all food-grade GRAS or approved food additives in accordance with US federal regulations. The raw materials used in fermentation conform to current Food Chemicals Codex specifications except for those that do not appear in the FCC. There are no ingredients based on milk, soy, or any of the named nine allergens in the Food Allergen Safety, Treatment, Education & Research Act (FASTER Act, 2021).

2.3.2.2 Inoculum (Seed)

A suspension of a pure culture of *E coli* BLASC cells 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 is subsequently used as inoculum for the seed fermentation.

2.3.2.3 Seed Fermentation

The inoculum is aseptically transferred to the seed fermenter containing seed fermentation medium. When a sufficient amount of biomass is developed (typically up to 8 hours), the content of the seed fermenter is used for inoculation of the main fermentation.

2.3.2.4 Main fermentation

The enzyme production takes place at this stage. The fermentation is operated in fed-batch mode. The production microorganism *E coli* BLASC grown in seed fermenter is aseptically transferred to the main fermenter containing the sterilized and cooled fermentation medium. After desired growth is achieved in the fermenter, isopropyl-β-D-thiogalactopyranoside (IPTG) is aseptically added to induce the synthesis of the enzyme, which is produced and

retained intracellularly. The fermentation process is continued for a predetermined time or until laboratory test data show that, the desired enzyme production has been obtained or that the rate of enzyme production has decreased below a predetermined production rate. When the desired enzyme level is reached, the fermentation is complete.

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

2.3.3 *RECOVERY*

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

During fermentation, the enzyme protein maltogenic amylase is produced and retained by the GMM *E. coli* BLASC, intracellularly. During recovery, the fermentation broth is subjected briefly to an elevated temperature with due consideration to enzyme stability and passed through a cell homogenizer to lyse the cells, this results in the release of the enzyme from the bacterial cells. The enzyme-containing fermentation medium is then separated from the biomass.

The steps of enzyme recovery include:

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

2.3.3.1 Primary Separation

Filter aids are added to promote biomass flocculation and filtration is carried out at a controlled pH using a horizontal filter press. This enables separation of the biomass and other insoluble from the soluble enzyme containing liquid. The primary separation is performed at defined pH and temperature ranges in order to minimize the loss of enzyme activity.

2.3.3.2 Concentration and centrifugation

The primary filtration step is followed by ultrafiltration and diafiltration of the filtrate from the primary recovery step to concentrate the enzyme containing filtrate and to reach the desired enzyme activity. Temperature and pH are controlled during this step. The filtered liquid is passed through a high-speed centrifuge to separate and remove other insoluble and partially soluble impurities.

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

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

2.3.3.4 Spray Drying

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

2.3.4 *FORMULATION AND PACKAGING*

Maltogenic amylase is sold as a powder preparation of different enzyme activities.

If required, the spray-dried unformulated concentrate powder (not less than 100,000 MAN U/g) is further formulated with GRAS or approved food additive, food grade formulating agents such as maltodextrin and adjusted to a declared enzyme activity.

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

Allergenicity of enzyme preparations

The maltogenic amylase is not, itself a known allergen (See section 6.2.3). Materials used in the fermentation process (inoculum, seed, and main fermentation) of maltogenic amylase are food-grade substances. There are no ingredients based on milk, soy, or any of the listed nine allergens used during fermentation process. (FASTER Act, 2021).

Moreover, no allergenic ingredients are added post fermentation in enzyme preparation, causing no allergenicity or labelling concerns. Allergen information for maltogenic amylase enzyme preparation is provided in Annex A.

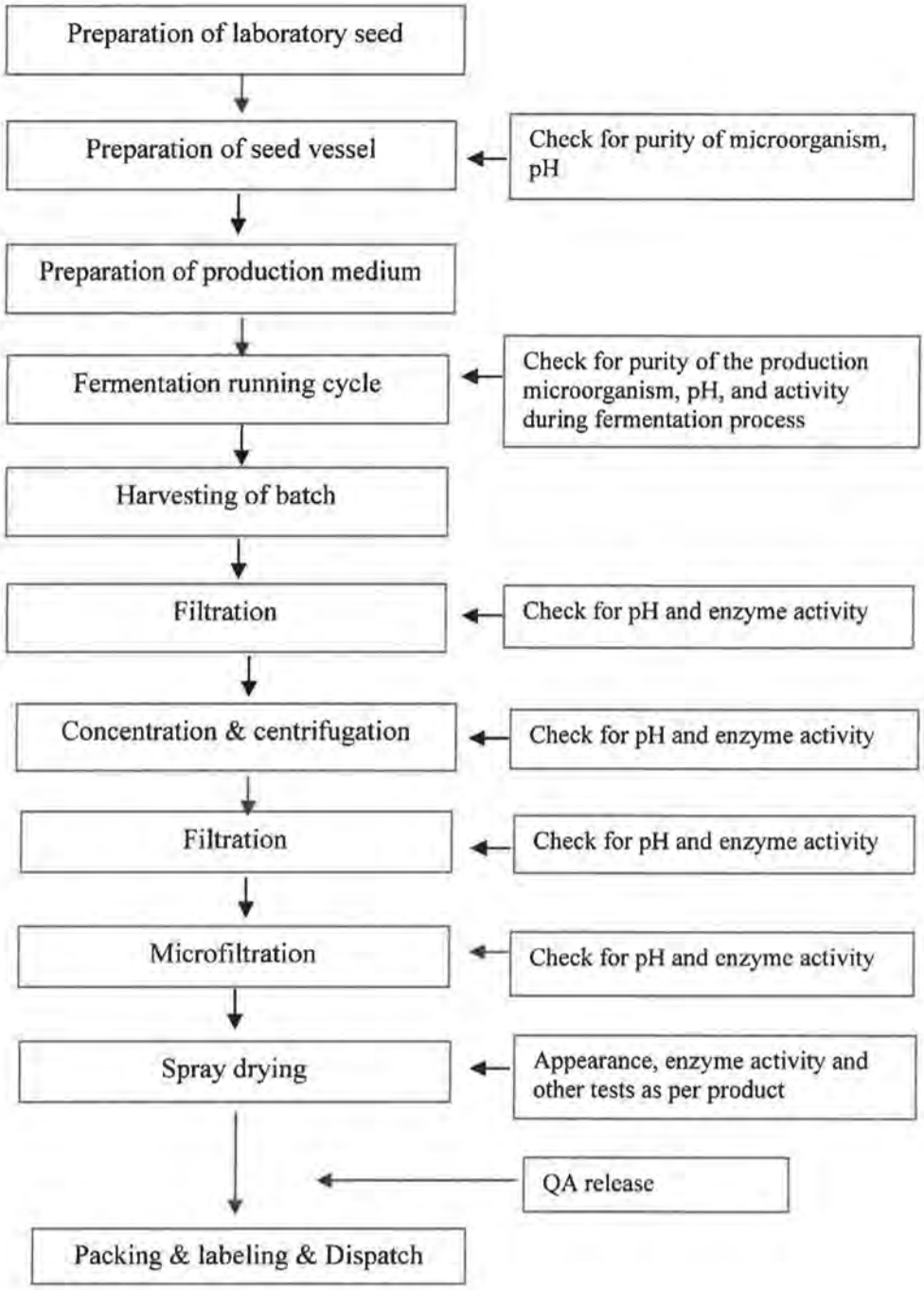


Fig.8: Manufacturing process of the maltogenic amylase from *E. coli* BLASC

2.3.5 *QUALITY CONTROL OF FINIHED PRODUCT*

The proposed processing aid complies with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (FAO/WHO, 2006a,b), as mentioned in the table below.

Test	Limits	Reference
Heavy metals	Not more than 30 mg/kg	AOAC 984.27, AOAC 999.10
Lead	Not more than 5 mg/kg	AOAC 984.27, AOAC 999.10
Salmonella spp.	Absent in 25 g of sample	Harmonized method (USP,EP and JP) and IP
Total coliforms	Not more than 30 per gram	Harmonized method (USP,EP and JP) and IP
Escherichia coli	Absent in 25 g of sample	Harmonized method (USP,EP and JP) and IP
Antimicrobial activity	Not detected	JECFA 2003 (FNP 52, Add. 11)

The specifications of the maltogenic amylase are described in Section 2.4.

The proof that the maltogenic amylase from *E. coli* BLASC complies with these specifications is shown by the analysis on three batches. Refer to Annex B for analyses of heavy metals, and section 6.2.2 for antimicrobial activity.

2.4 Product Specifications and Compositional Variability

2.4.1 *PRODUCT SPECIFICATIONS*

Specifications for maltogenic amylase preparation have been established by Advanced Enzyme Technologies Ltd. and are summarized in Table 1. All methods are standard method validated for the purpose and references are provided in the below table.

Table 1: Product specifications for maltogenic amylase from *E. coli* BLASC

Product specification	Advanced Enzyme Technologies Ltd.	
	Limits	Reference Method
Maltogenic amylase activity	Not less than 100,000 MAN U/g	Based on the monographs mentioned in the JECFA (1999) Korea Food Additive Code
Appearance/ Description	Light brown to brown colored powder with characteristics odor	Visual, olfactory
Moisture/ Loss on Drying	Not more than 10.0%	Indian Pharmacopoeia
Solubility	Soluble in water	Harmonized method (USP, EP and JP) and IP
Heavy metals	Not more than 30.0 ppm	AOAC 984.27, AOAC 999.10
Lead	Not more than 5.0 ppm	AOAC 984.27, AOAC 999.10
Total viable count	Not more than 10000 cfu/g	Harmonized method (USP,EP and JP) and IP
Total coliform	Not more than 30 cfu/g	Harmonized method (USP,EP and JP) and IP
E. coli /25g	Negative by test	Harmonized method (USP,EP and JP) and IP
Salmonella spp. /25g	Negative by test	Harmonized method (USP,EP and JP) and IP
Antimicrobial activity	Absent by test	JECFA 2003 (FNP 52, Add. 11)

2.4.2 *COMPOSITIONAL VARIABILITY*

Commercial enzymes, whether used in the production of food, feed, or for technological purposes, are biological isolates of variable composition. Apart from the enzyme protein, in question, microbial food enzymes also contain some substances derived from the producing microorganism and the fermentation medium. These constituents consist of organic material and inorganic salts. As established by JECFA (FAO/WHO, 2006 a,b), the percentages of these organic materials are summarized and expressed as Total Organic Solids (TOS)[1].

2.4.2.1 Quantitative Composition

The relative purity of maltogenic amylase from *E. coli* BLASC was measured, and the TOS values were calculated, in 3 batches after drying.

Table 2: Analysis of compositional variability of maltogenic amylase from *E. coli* BLASC

Batch no	031434[2]	071404	092101	Mean enzyme activity
Ash (%)	8.86	8.21	7.80	-
Water (%)	7.35	6.97	7.10	-
TOS (%)	83.79	84.82	85.10	-
Maltogenic amylase activity (MAN U/g)	144255	153647	146790	148230
U/mg TOS[3]	172.16	181.14	172.49	-
Unit Definition: One unit of enzyme activity is defined as the amount of enzyme that cleaves 1 µmol of maltotriose per minute under the given assay conditions.				

[1] The TOS value is an internationally accepted method to describe the chemical composition of commercial food enzymes (TOS% =100% – (Ash % + Water % + Diluent %). The ratio between the enzyme activity and TOS is an indication of the relative purity of the enzyme.
[2] The batch used for toxicity studies adheres to JECFA specifications for food enzyme(s).
[3] U/mg TOS = Activity U/mg x 100 / TOS %

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

Three batches of maltogenic amylase from *E. coli* BLASC were analyzed and the results were compared with food-grade specifications. As shown in Table 3, all tested batches complied with the product specifications, demonstrating the suitability of the production process.

Table 3: Analysis of compositional variability of maltogenic amylase from *E. coli* BLASC

Parameter	Specification	Batch		
		031434	071404	092101
Maltogenic amylase Activity	Not less than 100,000 MAN U/g	144255 MAN U/g	153647 MANU/g	146790 MAN U/g
Description	Light brown to brown colored powder having typical fermentative odor	Light brown to brown colored powder having typical fermentative odor	Light brown to brown colored powder having typical fermentative odor	Light brown to brown colored powder having typical fermentative odor
Solubility	Soluble in water	Soluble in water	Soluble in water	Soluble in water
Moisture/Loss on drying (%)	Not more than 10.0%	7.35%	6.97 %	7.10 %
Heavy Metal Analysis				
Heavy metals	Not more than 30 ppm	Complies	Complies	Complies
Lead	Not more than 5.0 ppm	Complies	Complies	Complies
Microbial Analysis				
Total viable count	Not more than 10000 cfu/g	Complies	Complies	Complies
Total Coliform	Not more than 30 cfu/g	Complies	Complies	Complies
E. coli	Absent in 25g	Complies	Complies	Complies
Salmonella spp.	Absent in 25g	Complies	Complies	Complies
Antimicrobial activity	Absent by test	Complies	Complies	Complies

2.5 Technical Effect

2.5.1 *MODE OF ACTION*

Maltogenic amylase hydrolyzes (1→4)-alpha-D-glucosidic linkages in starch polysaccharides to remove successive alpha-maltose residues from the non-reducing ends of the chains. It converts starch into maltose (dimer of glucose units) and maltotriose (trimer of glucose units) as the main hydrolysis product.

2.5.2 *USES*

In principle, the enzymatic conversions of starch with the help of maltogenic amylase can be of benefit in the processing of all foods and food ingredients, which naturally contain starch.

Baking:

During baking, the maltogenic amylase is added to the raw materials during the preparation of the dough. It is used to shorten the branched part of the amylopectin molecules during dough handling.

The stickiness of baked goods during processing is addressed by using maltogenic amylase, which produces linear oligosaccharides of 2–6 glucose residues. (e.g., maltose, maltotriose and maltotetraose). The linear oligosaccharides, thus produced, tend to increase the shelf life of bakery products by delaying retrogradation of the starch. (Marc et al., 2002).

The process flow chart of use and fate of maltogenic amylase in baking is shown below.

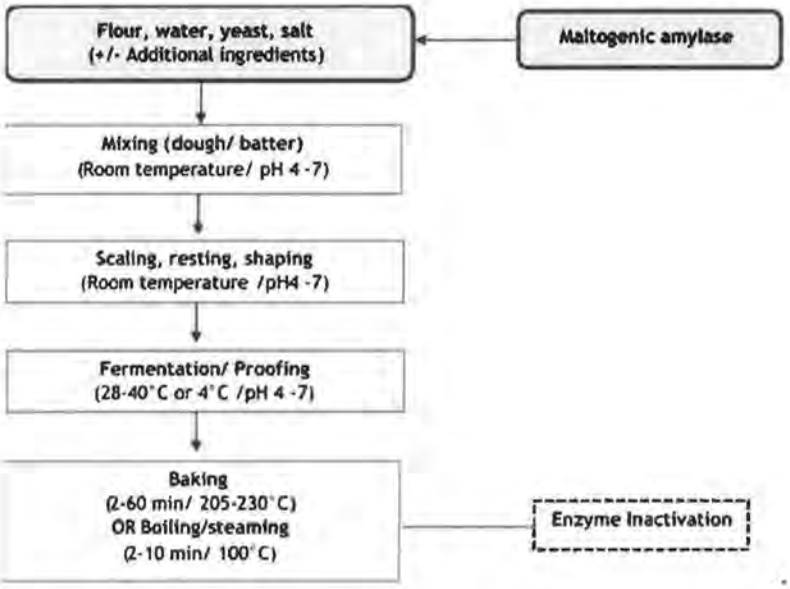


Fig. 9: Flow-chart - Use and fate of the maltogenic amylase in baking

Starch processing for the production of maltose/ glucose syrup:

In starch processing for the production of maltose/ glucose syrups, the food enzyme maltogenic alpha amylase is added to the saccharification step. The hydrolysis of starch results in faster and improved processing improved yields of high maltose syrup, and hydrolysis of maltotriose to maltose and glucose.

Production of high maltose syrup from starch generally involves liquefaction and saccharification, as in the production of glucose. However, in this process liquefaction reaction is terminated when the Dextrose equivalent (DE) reaches about 5-10 since a low DE value increases the potential for attaining high maltose content. Maltogenic alpha amylase hydrolyses α-1,4-oligosachharide links to predominantly yield maltose/glucose syrup.

The process flow chart of use and fate of maltogenic amylase in the production of maltose/ glucose syrup is shown below.

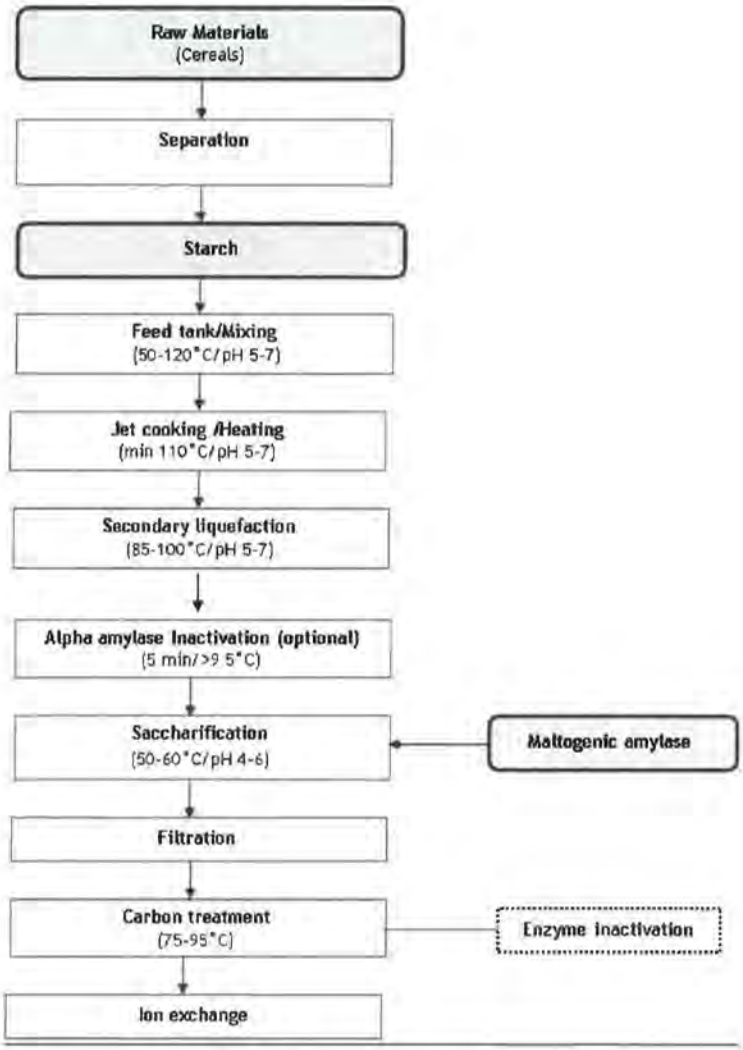


Fig 10: Flow-chart - Use and fate of maltogenic amylase in starch processing

Brewing:

In brewing processes, the maltogenic alpha amylase is added during mashing, before lautering or mash filtration. The enzyme hydrolyses the starch present in substrate into simple sugars, which allows yeast to work continuously during fermentation. The benefits of usc of maltogenic alpha amylase are improved yields due to the release of high amounts of maltose, decreased production time and wider choice of raw materials.

The process flow chart of use and fate of maltogenic amylase in brewing is shown below.

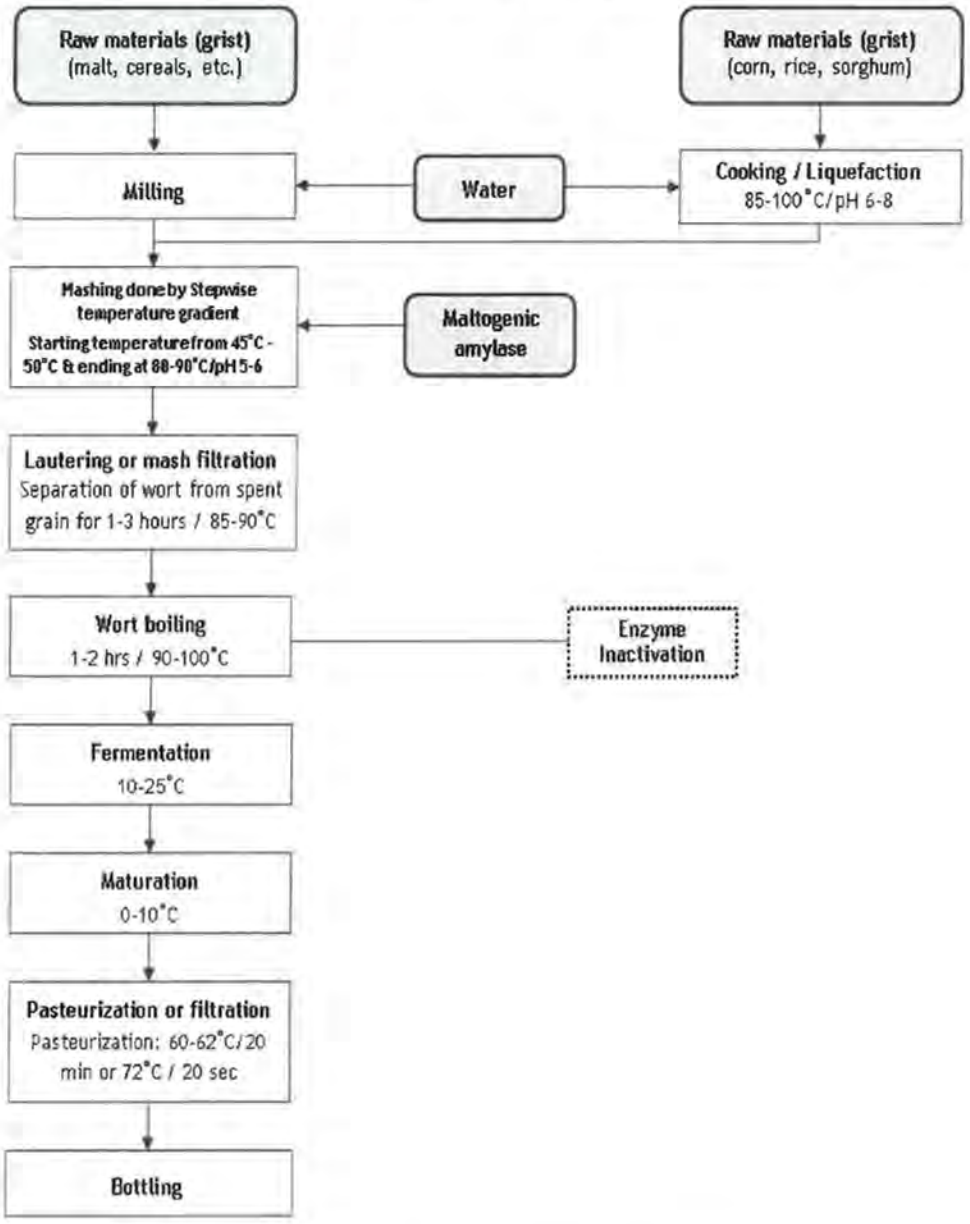


Fig 11: Flow-chart - Use and fate of the maltogenic amylase in brewing

2.5.3 *USE LEVELS*

Recommended use levels of food enzyme, maltogenic amylase are given in Table 4 based on food application.

Table 4: Recommended use levels

Application	Raw material (RM)	Recommended use levels (mg TOS/kg RM)	Maximal recommended use levels (mg TOS/kg RM)
Baking	Flour	0.85 – 8.47	8.47
Starch processing for production of maltose/ glucose syrup	Starch	12.71 – 33.89	33.89
Brewing	Malted barley/starch	6.78 – 16.95	16.95

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

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

Maltogenic amylase performs its technological function during food processing. Like the maltogenic amylase present in food raw materials and ingredients, it does not perform any technological function in the final food. The reasons why the maltogenic amylase does not exert enzymatic activity 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.

The fate of maltogenic amylase in various processes are explained in more detail below:

Baking:

During baking, maltogenic amylase is denatured and inactivated by heat during the baking step. Since the enzyme is inactivated, no residual enzyme activity remains in the finished product.

Starch processing for the production of maltose/ glucose syrup:

Post enzymatic action of maltogenic amylase, there are heating steps involved in the starch processing. The enzyme gets inactivated as the temperature increases at different stages in the process increases.

Brewing:

In brewing processes, the maltogenic amylase is added to different steps of the process (cooking/liquefaction, mashing, before lautering or mash filtration, after fermentation). At the end of the mashing, maltogenic amylase is inactivated by increasing the temperature of mash; further, it is also denatured in the consecutive lautering or mash filtration, wort-boiling steps.

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

Estimates of human consumption

The maltogenic amylase covered in this dossier is intended to be used in the baking process, brewing process, and starch processing for the production of maltose/ glucose syrups.

Due to the wide variety of applications, the most appropriate way to estimate human consumption in the case of food enzymes is using the Budget Method (Hansen, 1966; Douglass et al., 1997). This method enables to calculate a Theoretical Maximum Daily Intake (TMDI) based on certain conservative assumptions regarding physiological requirements for energy from food and the energy density of food rather than on food consumption survey data.

Table 5: Maximal level of food enzyme maltogenic amylase in final food(s)

Application		Raw material (RM)	Maximal recommended use level (mg TOS/kg RM)	Example Final food (FF)	Ratio RM/FF	Maximal level in FF (mg TOS/ kg food)
Solid	Baking	Flour	0.85 – 8.47	Bread	0.667	5.648
Liquid	Starch processing for the production of maltose/ glucose syrup	Starch	12.71 – 33.89	Sweetener	1.0	33.892
	Brewing	Malted barley/starch	6.78 – 16.95	Beer	0.1667	2.824

For the calculation of the TMDI, the maximum use levels are chosen. Furthermore, the calculation takes into account how much food or beverage is obtained per kg raw material (The Fresh Loaf Baker's Handbook; Sweetness from starch, FAO Corporate document repository; Barely Malt Beer, Agribusiness Handbook -for information on how these ratios were obtained) and it is assumed that all the TOS will end up in the final product.

The Total TMDI can be calculated on basis of the maximal values found in food and beverage [in the above case, Baking and Starch processing for the production of maltose/ glucose syrup], multiplied by the average consumption of food and beverage/kg body weight/day. Consequently, the Total TMDI will be:

TMDI in solid food (mg TOS/kg body weight/day)	TMDI in beverage (mg TOS/kg body weight/day)	Total TMDI (mg TOS/kg body weight/day)
5.648 x 0.0125 = **0.0706**	33.892 x 0.025 = **0.8473**	**0.9179**

It should be stressed that this Total TMDI is based on conservative assumptions and represents a highly exaggerated value because of the following reasons:

- It is assumed that the producer of the above-mentioned foodstuffs and beverages use the specific enzyme maltogenic amylase.
- It is assumed that the producer applies the highest use level per application;
- For the calculation of the TMDI's in food as well as in beverages, only those foodstuffs and beverages were selected containing the highest theoretical amount of TOS. Thus, foodstuffs and beverages containing lower theoretical amounts were not taken into account;
- It is assumed that the amount of TOS does not decrease as a result of the food production process;
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed daily over the course of a lifetime;
- Assumptions regarding food and beverage intake of the general population are overestimates of the actual average levels (Douglass *et al.*, 1997).

The no observed adverse effect level (NOAEL) dose for maltogenic amylase from *E coli* BLASC, concluded from the 90-day toxicity study (see Section 6.4) is 837.9 mg TOS/kg body weight/day (Considering 83.79% TOS), corresponds to 144,255 MANU/ /kg body weight/day. Acceptable Daily Intake (ADI) concluded from the NOAEL dose, is 586.53 mg TOS/person/day or 100978.5 MANU/person/day (ADI = NOAEL x 70/100, where body weight of a healthy individual is considered 70 kg and a safety factor of 100 is considered for determination of dose for healthy human adults and children).

Based upon the above calculation, the maximum Estimated Daily Intake (EDI) is 64.25 mg TOS per day, which is much lower than the ADI, i.e. 586.53 mg derived from the NOAEL 837.9 mg, obtained from the 90-day chronic oral toxicity study.

Safety Margin

The Margin of Safety (MoS) for human consumption is 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 **0.9179 mg TOS/kg body weight/day.**

Margin of Safety (MoS) = 837.9/0.9179 = 912.84, i.e., 913

As is explained above, the TMDI is highly exaggerated. Moreover, the NOAEL was based on the highest dose administered, and is therefore to be considered as a minimum value.

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

There are no self-limiting levels of use of maltogenic amylase from *E. coli* BLASC in food applications, as the enzyme should be used in accordance with good manufacturing practices.

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

The statutory basis for our conclusion of GRAS status in the notice is scientific procedures rather than on common use in food prior to 1958.

Part 6: 21 CFR 170.250: Narrative

6.1. Safety of Donor and Production Strain

The microorganism used for the production of maltogenic amylase is a non-pathogenic, non-toxigenic strain of *E coli* BLASC. The microorganism has been genetically modified. The recombinant production strain *Escherichia coli* (strain BLASC) is characterized as *Escherichia coli* BL21 (DE3) using 16S rRNA as a phylogenetic marker.

The production strain *E. coli* BLASC is generated from the host strain *E. coli* SAML27 [Derivative of BL21(DE3)] by transforming the plasmid pTA-AmyM. *E. coli* SAML27 is a derivative of *E. coli* BL21 (DE3) that is a non-pathogenic laboratory strain. *E. coli* strain BL21 (DE3) is commonly used as a production host to produce various recombinant proteins (Rosano et al., 2014; Gopal et al., 2013; Huang et al., 2012; Liu et al., 2015, Jia et al., 2016)

The *E. coli* B, C, and W strains and their derivatives are all classified as risk group 1 organisms in biosafety guidelines (Bauer et al., 2007). Bioinformatic observations have demonstrated that *E. coli* BL21 does not contain functional/intact gene sequences encoding an O antigen polysaccharide (Jeong et al., 2009). It has also been observed that *E. coli* "BL21, did not have the well-recognized pathogenic mechanisms required by strains of *E. coli* causing the majority of enteric infections". The absence of toxin production by *E. coli* BL21 is demonstrated in the feeding studies, where oral and/or intraperitoneal administration of *E. coli* BL21 to mice, 1-day-old chicks, and sheep were without evidence of intolerance or toxicity (Chart et al., 2000).

The entire genome of *E. coli* BL21 (DE3) was characterized by Jeong et al. (2009), and the whole genomic sequence of the strain was compared in detail to the genomes of strains *E. coli* K-12 (MG1655) and *E. coli* REL606 using bioinformatic analyses (Studier et al., 2009). Consistent with the long history of safe use of these strains in the laboratory, and the history of safe use of *E. coli* K-12 in the production of food additives and pharmaceutical preparations, known *E. coli* enterotoxins or related pathogenic determinants were not identified by the authors.

The safety aspect of recombinant protein produced by *E. coli* strains was assessed for its presence of toxins through the genetic alignment between common *E. coli* strains in recombinant work and toxin genes. Stx, LT and ST toxin genes were used for evaluation. The BL21(DE3) and K-12 MG1655 *E. coli* strains were used as a representative in the alignment process, which generates non-overlapping alignment. This concluded the absence of toxin gene in these strains. Therefore, expressing the recombinant protein, especially therapeutic protein, in *E. coli* was considered to be safe against the toxin (Maksum et al., 2020).

Also, a significant number of short-term and subchronic toxicity evaluations have been conducted with recombinant proteins produced by *E. coli* BL21 (DE3) without evidence of confounding toxicity from toxins originating from the organism during fermentation

(Mathesius et al., 2009; Guimarães et al., 2010; Quemada et al., 2010). This extensive history of use for the production of recombinant proteins further supports the conclusion that *E. coli* BL21(DE3) can be concluded to be a non-toxicogenic strain.

Further, the donor microorganism - *Geobacillus stearothermophilus* - is a member of *Bacillus* species group, which has a long history of safe use in industrial-scale enzyme production and can be considered as safe for the production of enzymes for food as well as feed processing and numerous other industrial applications. Importantly, EFSA has assigned *Geobacillus stearothermophilus* a "Status of Qualified Presumption of Safety" (QPS) (EFSA 2007, 2020a).

6.2 Safety of Enzyme

Enzymes, including maltogenic alpha amylase, have a long history of safe use in food. Since 1960s, alpha-amylases have been used extensively in various industrial food applications for the hydrolysis of starch (Marc et al., 2002; Pence, 1953). Enzymes, in general, are widely used in bakery products because they improve volume, flavour, aroma, softness, crumb structure, and increase shelf life (Lagrain et al., 2008; Guy & Sahi, 2006). Maltogenic alpha amylase is used in baking to allow for extensive hydrolysis of crystallisable amylopectin, preventing its recrystallization (retrogradation) during storage. This allows for the conservation of the soft crumb characteristics and prolonging the shelf life (Goesaert et al., 2009). Microbial amylases have replaced chemical hydrolysis to a large extent in the starch processing industry (Gupta et al., 2003; Marc et al., 2002). Maltogenic alpha amylase preparations from various sources are widely authorised as processing aid in, e.g., Australia and New Zealand, Brazil, Canada, China, Denmark, France.

Also, FDA, had no questions regarding the conclusion that maltogenic amylase enzyme preparation is GRAS under the intended conditions of use (GRN 746, GRN 751, GRN 842). Moreover, EFSA has evaluated maltogenic amylase from *E. coli* BLASC, subject of present GRAS assessment, as a processing aid and concluded that the enzyme does not raise safety concerns under the intended condition of use (EFSA Journal, 2019).

6.2.1 INFORMATION ON ANY SIGNIFICANT SIMILARITY BETWEEN THE AMINO ACID SEQUENCE OF THE ENZYME AND THAT OF KNOWN PROTEIN TOXINS

Bacterial toxins are virulence factors that manipulate host cell functions and take over the control of vital processes of living organisms to favour microbial infection. To confirm that the maltogenic alpha amylase is non-toxic, a sequence homology assessment of the maltogenic alpha amylase enzyme with known toxins was conducted. The amino acid sequence was aligned with the known toxin sequences available in the "Database of Bacterial ExoToxins for Human (DBETH)" (Chakraborty et al., 2012). The database consists of structures, interaction networks and analytical results for 229 exotoxins, from 26 different humans' pathogenic bacterial genus. All toxins are classified into 24 different Toxin classes. The amino acid sequence of the maltogenic alpha amylase provided in the section 2.1.2 was used as input for the search.

Maltogenic amylase protein did not show homology with known toxin protein sequences described in database sequences.

6.2.2 *ANTIMICROBIAL ACTIVITY OF MALTOGENIC AMYLASE ENZYME PRODUCED BY A RECOMBINANT E. COLI STRAIN*

The maltogenic alpha amylase from *E. coli* BLASC was evaluated for its antimicrobial activity following CLSI (2012) guidelines and EFSA (2008 & 2009). Three batches of maltogenic alpha amylase were checked for their antimicrobial activity against the five selected microorganisms as recommended by EFSA [*Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 & *Enterococcus faecalis* ATCC 29212]. Additionally, three more microorganisms [*Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739 & *Staphylococcus epidermis* ATCC 12228] were included in the study as described in United States Pharmacopoeia (USP, 2008). Maltogenic alpha amylase showed an absence of antimicrobial activity against all the test microorganisms.

Further, the maltogenic amylase covered in this dossier and produced by *E coli* BLASC does not contain any detectable amount of full-length recombinant DNA as determined by the absence of amplicon for a full-length recombinant gene of maltogenic amylase. This confirmed the absence of recombinant DNA in the product (Refer to section 2.2.7 for details).

6.2.3 *ALLERGENICITY*

The allergenic potential of the maltogenic amylase was assessed following the pepsin digestion of the maltogenic amylase and bioinformatics analyses of its amino acid sequence.

6.2.3.1 Evaluation of allergenicity of the maltogenic amylase following pepsin digestion method

Allergenicity of the maltogenic amylase from *E. coli* BLASC was evaluated following pepsin digestion method. The pepsin digestion study is based on the recommendations made by the Joint FAO/WHO Expert Consultation (2001). The protocol used for this assay is based on the detailed study undertaken by Thomas et al. (2004), which in turn takes into consideration the guidelines provided by the Codex Alimentarius Commission (2003).

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

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

6.2.1.2 Sequence homology of the maltogenic amylase protein with the known allergens

Allergenic potential of the maltogenic amylase was assessed following the bioinformatics analyses of its amino acid sequence. Over the last decade, bioinformatics methods have been widely used for collecting, storing, and analyzing molecular and/or clinical information of

importance for allergy. In order to address the allergenicity, information obtained from bioinformatics, coupled with experimental data, wherever necessary, is the approach postulated by the joint Food and Agriculture Organization and World Health Organization (FAO/WHO) Expert Consultation on Allergenicity of Foods Derived from Biotechnology. This approach takes into account the evidence derived from several types of information and data since no single criterion is sufficiently predictive (Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology, 2001).

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 enzyme, using a window of 80 amino acids and a suitable gap penalty, or (b) A stretch identity of 6-8 contiguous amino acids.

The maltogenic amylase sequence (as presented in section 2.1.2) was analyzed using the Food Allergy Research and Resource Program (FARRP) database.[4] Bioinformatics searches showed that maltogenic amylase from *E. coli* BLASC, showed a maximum of 41.28%, identity with allergen Asp o 21 over a sliding window of 80 amino acids or full length. Although the enzyme protein(s) showed an identity of 35% or more with some known allergen over an 80 amino acid sliding window, the percentage identity (PID) was not more than 50%. On conducting a full length FASTA alignment, the PID was less than 50%; in fact, the PID is only 27.9%. Additionally, no hits were obtained for a match of 8 contiguous amino acids. This analysis, based on the comparison of the maltogenic amylase with the amino acid sequences of known allergen, showed no allergenicity concern.

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 including maltogenic amylase. The investigation comprised enzymes produced by wild-type, genetically modified strains as well as protein engineered variants. The study comprised of 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp wherein 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.

The 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 minuscule quantities of the enzyme(s) in the final food. Goodman et al. (2008) discuss that a high concentration generally equals a higher risk of sensitization, whereas a low level in the final food equals a lower risk. Additionally, it must be noted that the food enzyme protein

[4] https://farrp.unl.edu/

undergoes denaturation under various conditions of food processing, resulting in the loss of its tertiary structure. In general, these alterations in conformation are associated with a 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, 2012; Kikuchi et al., 2006]. Additionally, residual enzyme(s) still present in the final food will be subjected to digestion in the gastro-intestinal system, which further reduces the risk of enzyme allergenicity. It is believed that small protein fragments resulting from digestion are less likely to be allergenic (FAO/WHO, 2001; Goodman et al., 2008).

The results presented above suggest that the maltogenic amylase enzyme is readily digested by the pepsin. Bioinformatics analyses do not show any similarity of maltogenic amylase amino acid sequence with a known allergen. Based upon the evidence presented above, it is highly unlikely that the maltogenic amylase enzyme presents allergenicity concerns as a result of the intended uses.

6.2.4 *REGULATORY HISTORY AND LEADING ENZYME PUBLICATIONS ON SAFETY OF THE ENZYME*

Maltogenic amylase has a long history of use in food processing. Foods processed with maltogenic amylase are known to be consumed safely by the general human population. There are precedence of regulatory recognition of maltogenic amylase. The following summarizes regulatory recognition for maltogenic amylase.

- Food Standards Australia New Zealand (FSANZ) categorized maltogenic alpha amylase from *Bacillus* spp. (i.e. *B. subtilis* containing the gene for maltogenic α-amylase isolated from *Geobacillus stearothermophilus*) as a processing aid under Australia New Zealand Food Standards Code - Standard 1.3.3.

- Legifrance (French Government) approved use of maltogenic exo-alpha amylase from genetically modified strains of *B. subtilis* (OC, SM, SO, DS 67348) containing the alpha amylase gene from *B. stearothermophilus* and genetically modified strain of *B. licheniformis* (MDT06-221) containing the synthetic gene encoding a maltogenic exo-alpha amylase from *Geobacillus stearothermophilus* as a technological aid in the manufacture of certain foodstuffs (Order of October 19, 2006).

- US FDA has issued "no questions" letters for the maltogenic alpha amylase from *Geobacillus stearothermophilus* produced by strains of *B. subtilis* and *Saccharomyces cerevisiae* and considered as GRAS under the intended conditions of uses (GRN 746, GRN 751, GRN 842, GRN 974, and GRN 975).

- Health Canada has classified amylase (maltogenic) from different strains of *B. subtilis* and *B. licheniformis* as a food additive under the list of permitted food enzyme.

- National Health Commission (NHC) of China has approved maltogenic amylase from *Bacillus subtilis* as a processing aid (Chinese Standards for Food Additives - GB2760-2015).

- EFSA has evaluated maltogenic amylase from *E. coli* BLASC as a processing aid (EFSA Journal, 2019) and found no safety concern under intended conditions of use. Similarly, maltogenic amylase from genetically modified strains of *Bacillus subtilis* (strain NZYM-SM) [EFSA, 2018a], (strain NZYM-SO) [EFSA, 2018b], *Bacillus licheniformis* (strain DP-Dzr50) [EFSA, 2020b] was also evaluated by EFSA and no safety concerns were reported.

6.3 Safety of Manufacturing Process

The maltogenic amylase described in this dossier is manufactured as per current Good Manufacturing Practice (cGMP) in accordance with 21CFR§110 and 21CFR§117 and the principles of Hazard Analysis and Critical Control Points (HACCP). The manufacturing facility is ISO 9001, ISO 22000 (Food Safety Management System) and GMP certified. The analysis of three (3) non-consecutive batches demonstrates that the process is capable of producing product that meets the established specifications.

6.4 Safety of Maltogenic amylase —Oral Toxicity and Genotoxicity Studies

Maltogenic amylase from *E. coli* BLASC, the notified enzyme, has been investigated in a series of systemic and genetic toxicity studies complied with OECD Guidelines and conducted according to the principles of Good Laboratory Practice (GLP) as published by the OECD (ENV/MC/CHEM (98)17). The summary of toxicity studies is provided in Annex C.

Acute oral toxicity test (OECD Test No. 423, 2001): Using the step-wise method, 2 groups of n=3 female Wistar rats aged 9 weeks and weighing 143.0-158.0 g were dosed via gavage with 2000 mg preparation/kg bw and observed for 14 days. No indications of toxicity were reported. (Annex C)

Repeated-dose 90-day oral toxicity test (OECD Test No. 408, 1998): Four groups of Wistar rats (10/sex/group), 7 weeks old and weighing 117-156 g (males, mean = 136.50 g) and 101-139 g (females, mean = 120.00 g) were assigned to receive daily oral gavage of doses of 0, 250, 500, and 1000 mg /kg bw for 90 days. Additional, groups of 5 rats/sex receiving 0 or 1000 mg of enzyme preparation/kg bw/day were assigned to 28-day recovery groups. Rats were examined daily for signs of toxicity, morbidity, and mortality. They were subjected to detailed clinical examinations at day 0 and weekly thereafter during the treatment and recovery period. Ophthalmic examinations were performed on the control and high-dose rats at the beginning and end of dosing. At week 13, all animals were assessed for sensory reactivity, grip strength, and motor activity. Feed consumption and body weight were recorded weekly. Blood and urine samples were collected for hematology and clinical chemistry

analysis at the end of dosing and after recovery. All animals were subjected to necropsy and weights of kidneys, liver, adrenals, testes, epididymis, uterus, thymus, spleen, brain, ovaries, and heart were recorded. Histological evaluations were performed on all tissues from control and high-dose rats.

There was no mortality and no clinical abnormalities in rats treated at any dose. Ophthalmological examination revealed no abnormalities, nor did the neurotoxic assessment. There was no effect on feed intake or body weight gain, hematological or biochemical parameters, absolute or relative organ weights and no histopathology. The no observed adverse effect level (NOAEL) of maltogenic amylase from *E. coli* BLASC preparation in the Wistar rat, following oral administration for 90 days, was the highest dose tested, 1000 mg /kg bw/day (837.9 mg TOS /kg bw) providing 144255 MAN U/kg bw/day (Annex C).

Bacterial reverse mutation test—Ames assay (OECD Test No. 471, 1997): The test was conducted using *Salmonella typhimurium* tester strains TA97a, TA98, TA100, TA102, and TA1535 in the presence and absence of S9 metabolic activation. The test was conducted in triplicate at concentrations of 0, 50, 150, 500, and 5000 µg/plate. No significant increase in the number of histidine revertant colonies was reported, and it is concluded that, under the conditions of this study, maltogenic amylase from *E. coli* BLASC is non-mutagenic (Annex C).

***In vitro* mammalian chromosomal aberration test in human lymphocytes (OECD Test No. 473, 1997):** Cultures of human peripheral blood lymphocytes were exposed to maltogenic amylase from *E. coli* BLASC at concentrations of 0, 500, 1500 and 5000 µg/ml in the presence and absence of metabolic activation system for 3 or 24 hours. No significant concentration-related increase was reported in the incidence of structural chromosome aberrations at any tested concentration, and it was concluded that maltogenic amylase from *E. coli* BLASC is non-clastogenic in the presence and absence of microsomal enzymes (Annex C).

Based on the present toxicity data, it can be concluded that the maltogenic amylase enzyme preparation does not exhibit adverse toxicological effects under the experimental conditions described.

6.5 Decision Tree

The safety of maltogenic amylase from *E. coli* BLASC has also been established using the decision tree for evaluating the safety of microbial enzyme preparations used in food processing (Pariza et al., 2001)

1. Is the production strain genetically modified? **YES**

If yes, go to 2. If no, go to 6.

2. Is the production strain modified using rDNA techniques? **YES**

If yes, go to 3. If no, go to 5.

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

The maltogenic amylase 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. If no, go to 3b.

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. If no, go to 3d.

3e. Is all other introduced DNA well-characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade 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. If no, go to 12.

4. Is the introduced DNA randomly integrated into the chromosome? **NO**,

The introduced DNA (maltogenic amylase gene) is present in the plasmid, which is extrachromosomal.

If yes, go to 5. If no, 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 is a derivative of *E. coli* BL21 (DE3), which derived, is from the B lineage known as safety strains.

If yes, the test article is ACCEPTED.

6.6 Safety Assessment and GRAS Determination

This section presents an assessment that demonstrates that the intended use of maltogenic amylase from *E. coli* BLASC is safe and is GRAS based on scientific procedures.

This safety assessment and GRAS determination involves two steps. In the first step, the safety of the intended use of maltogenic amylase from *E. coli* BLASC is demonstrated. Safety is established by demonstrating a reasonable certainty that the exposure of consumers to maltogenic amylase under its intended conditions of use is not harmful. In the second step, the intended use of maltogenic amylase from *E. coli* BLASC is determined to be GRAS by demonstrating the safety of production organism and enzyme under its intended conditions of use is generally recognized among qualified scientific experts and is based on publicly available and accepted information.

The regulatory framework for establishing whether the intended use of a substance is GRAS, in accordance with Section 201(s) of the Federal Food Drug and Cosmetic Act, is set forth under 21 CFR §170.30. This regulation states that general recognition of safety may be based on the views of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. A GRAS determination may be made either: 1) through scientific procedures under §170.30(b); or 2) through experience based on common use in food, in the case of a substance used in food prior to January 1, 1958, under §170.30(c). This GRAS determination employs scientific procedures established under §170.30(b).

A scientific procedure GRAS determination requires the same quantity and quality of scientific evidence as is needed to obtain approval of the substance as a food additive. In addition to requiring scientific evidence of safety, a GRAS determination also requires that this scientific evidence of safety be generally known and accepted among qualified scientific experts. This "common knowledge" element of a GRAS determination consists of two components:

1. Data and information relied upon to establish the scientific element of safety must be generally available; and
2. There must be a basis to conclude that there is a consensus among qualified experts about the safety of the substance for its intended use.

The criteria outlined above for a scientific-procedures GRAS determination are applied below in an analysis of whether the intended use of maltogenic amylase from *E. coli* BLASC is safe and is GRAS.

6.6.1 *EVIDENCE OF SAFETY*

As noted in the sections above, the production organism *E. coli* BLASC, as well as the enzyme maltogenic amylase derived from it, are well characterized and safe for intended usage. Publicly available authorizations by various regulatory authorities, safety evaluations published in the peer-reviewed scientific literature, reviews by qualified experts, corroborated by Advanced Enzymes' unpublished safety studies, support such a conclusion.

The microorganism used for the production of maltogenic amylase is a non-pathogenic, non-toxigenic strain *E coli* BLASC. The production strain does not produce secondary metabolites of toxicological concern to the human. The production strain *E. coli* BLASC is generated from the host strain *E. coli* SAML27 which is a derivative of *E. coli* BL21 (DE3), a non-pathogenic laboratory strain. *E. coli* strain BL21 (DE3) is commonly used as a production host to produce various recombinant proteins (Rosano et al., 2014; Gopal et al., 2013; Huang et al., 2012; Liu et al., 2015; Jia et al., 2016). The *E. coli* B, C, and W strains and their derivatives are all classified as risk group 1 organisms in biosafety guidelines (Bauer et al., 2007).

The maltogenic amylase produced by the *E. coli* BLASC was evaluated for antimicrobial activity and the absence of antimicrobial activity was recorded against all the test micro-organisms. In addition, heavy metals in the enzyme preparations were well within the specification and below the detection limits of the analytical methods. Maltogenic amylase protein did not show homology with known toxin protein sequences described in DBETH database sequences. No indications of toxicity were found in acute and repeated-dose studies of oral toxicity or genotoxicity assays of maltogenic amylase obtained from *E coli* BLASC. Pepsin digestion analysis and bioinformatics studies on maltogenic amylase from *E. coli* BLASC showed that it was a labile protein and did not share homology with known allergens. Therefore, maltogenic amylase does not pose a concern in terms of food allergy.

Further, the maltogenic amylase as described in this dossier and produced by *E. coli* BLASC does not contain the production microorganism and any detectable amount of full-length recombinant DNA as determined by the absence of amplicon for full-length recombinant gene of maltogenic amylase.

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

Using the decision tree of Pariza and Johnson (2001), maltogenic amylase from *E. coli* BLASC enzyme preparation was determined to be acceptable for the proposed uses. Based on a worst-case scenario, the maximum daily intake is 0.9179 mg TOS/day which is lower than the Acceptable Daily Intake (ADI) derived from the NOAEL from the 90-day subchronic oral toxicity study i.e. 586.53 mg TOS per day. Therefore, the use of maltogenic amylase in the baking process, brewing process, and starch processing is not expected to result in adverse effects in humans. A safety margin of 913 exists between the established NOAEL and the estimated worst-case maximum daily human exposure level.

Based upon the totality of the evidence presented in this notice, Advanced Enzyme has determined by scientific procedures that the maltogenic amylase from *Geobacillus stearothermophilus* produced by *Escherichia coli* BLASC and manufactured according to the principles of cGMP is Generally Recognized As Safe for the intended processing aid uses in food.

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

7.1 Bibliography-Regulations / Reports by Important Agencies

Amraini SZ, Ariyani LP, Hermansyah H, Setyahadi S, Rahman SF, Park DH, , Misri G (2017). Production and characterization of cellulase from *E. coli* EgRK2 recombinant based oil palm empty fruit bunch. Biotechnol Bioproc E 22: 287–295

Arrêté du 19 Octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la fabrication de certaines denrées alimentaires; (2020)

Australia New Zealand Food Standards Code - Standard 1.3.3 - Schedule -18 Processing Aids (September, 2020)

Barley Malt Beer, Agribusiness Handbook; FAO Investment Centre Division (2009) http://www.fao.org/3/a-i1003e.pdf

Bauer A., Dieckmann S., Ludwig W., Schleifer K. (2007). Rapid identification of Escherichia coli safety and laboratory strain lineages based on Multiplex-PCR; FEMS Microbiol Lett 269 (2007) 36–40

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

Chakraborty A., Ghosh S., Chowdhary G., Maulik U., and Chakrabarti S. ,DBETH: A Database of Bacterial Exotoxins for Human. Nucleic Acids Res. 2012 Jan; 40

Chart H., Smith H R, Ragione R M, Woodward M J, An investigation into the pathogenic properties of *Escherichia coli* Strains BLR, BL21, DH5alpha and EQ1. J Appl Microbiol. 2000 Dec;89 (6):1048-58

Chinese Standards for Food Additives - GB2760-2015: National Food Safety Standard for Food Additive Use, implemented on May 24, 2015

CLSI (2012). Clinical and Laboratory Standards Institute: Performance standards for Antimicrobial disc susceptibility tests; Approved standards XI Edition.M02-11, Vol. 32 (1). Wayne, USA.

Douglass JS, Barraj 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

EFSA (2007) 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

EFSA (2008). Update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance: Prepared by the panel on additives and products or substances used in animal feed. The EFSA Journal 732: 1-15.

EFSA (2009). EFSA Guidance prepared by the Scientific Panel of Food Contact Material, Enzymes, Flavourings and Processing Aids on the Submission of a Dossier on Food Enzymes. The EFSA Journal 1305: 1-26.

EFSA Journal (2011), Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use. EFSA Journal 9(6): 2193

EFSA Journal (2014a) EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Technical Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition. EFSA Journal 2014;12 (5):3665.

EFSA Journal (2014b) Scientific Opinion on the safety and efficacy of concentrated liquid L-lysine (base), concentrated liquid L-lysine monohydrochloride and L-lysine monohydrochloride technically pure produced using *Escherichia coli* (FERM BP-11355) for all animal species based on a dossier submitted by Ajinomoto Eurolysine S.A.S , EFSA Journal 2014;12(11):3895

EFSA Journal (2018a), Safety evaluation of the food enzyme maltogenic amylase from a genetically modified *Bacillus subtilis* (strain NZYM-SM), EFSA Journal 2018;16(5):5171

EFSA Journal (2018b), Safety evaluation of the food enzyme maltogenic amylase from a genetically modified *Bacillus subtilis* (strain NZYM-SO), EFSA Journal 2018;16(11):5478

EFSA Journal (2019), Safety evaluation of the food enzyme maltogenic amylase from genetically modified *Escherichia coli* (strain BLASC), EFSA Journal 2019;17(5):5769

EFSA Journal (2020a) Scientific Opinion on the update of the list of QPS recommended biological agents intentionally added to food or feed as notified to EFSA (2017–2019). EFSA Journal 2020;18 (2):5966

EFSA Journal (2020b), Safety evaluation of the food enzyme maltogenic amylase from the genetically modified *Bacillus licheniformis* strain DP-Dzr50, EFSA Journal 2020;18(1):5972

Food Allergy Safety, Treatment, Education, and Research Act of 2021 (FASTER Act of 2021)

FAO/JECFA (2006a). Joint FAO/WHO Expert Committee on Food Additives Monographs: Combined compendium of food additive specifications, Volume 4, pp112

FAO/WHO (2006b). General Specifications and Considerations for Enzyme Preparations Used in Food Processing. Compendium of food additive specifications, sixty-seventh meeting. FAO JECFA Monographs 3, 2006, 63-67; (2006)

FARRP / AllergenOnline | http://www.allergenonline.org/databasefasta.shtml

Ferreira R.d.G, Azzoni AR, and Freitas S. 2018. Techno-economic analysis of the industrial production of a low-cost enzyme using *E. coli*: the case of recombinant β-glucosidase. Biotechnol Biofuels 11: 81

Goesaert, H., Leman, P., Bijttebier, A., & Delcour, J. A. (2009). Antifirming effects of starch degrading enzymes in bread crumb. Journal of Agricultural and Food Chemistry, 57(6), 2346-2355

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

Gopal GJ, Kumar A. Strategies for the production of recombinant protein in *Escherichia coli*. Protein J. 2013; 32(6):419–425

Guimarães LM, Farias DF, Muchagata RC, de Magalhães MQ, Campello CC, Rocha TL et al. 2010. Short-term evaluation in growing rats of diet containing Bacillus thuringiensis Cry1Ia12 entomotoxin: nutritional responses and some safety aspects. J Biomed Biotechnol doi:pii: 630267. 10.1155/2010/630267

Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B (2003) Microbial a-amylases:a biotechnological perspective. Process Biochemistry, 38, 1599-1616

Guy, R. C. E., & Sahi, S. S. (2006). Application of a lipase in cake manufacture. Journal of the Science of Food and Agriculture, 86(11),1679-1687

Hansen SC; Acceptable daily intake of food additives and ceiling on levels of use. (1966); Food Cosmet Toxicol; 4; 427–432

Health Canada, Food and Drug Regulations, C.R.C., c. 870, Section B.16.100, Table V. List of Permitted Food Enzymes (Lists of Permitted Food Additives) (2020)

Huang C-J, Lin H, Yang X. Industrial production of recombinant therapeutics in *Escherichia coli* and its recent advancements. J Ind Microbiol Biotechnol. 2012;39(3):383–399.

JECFA monograph (1999), Maltogenic amylase from *Bacillus stearothermophilus* expressed in *Bacillus subtilis*

Jeong H., Barbe V., Lee CH., Vallenet D. et al, Genome sequences of *Escherichia coli* B strains REL606 and BL21(DE3) J Mol Biol. 2009 Dec 11;394(4):644-52

Jia B, Jeon CO. High-throughput recombinant protein expression in *Escherichia coli*: current status and future perspectives. Open Biol. 2016;6:8

Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology; 22 – 25 January 2001; Topic 5: Stability of Known Allergens (Digestive and Heat Stability) |

Kamionka M. 2011. Engineering of therapeutic proteins production in *Escherichia coli*. Curr Pharm Biotechnol. 12(2):268–74

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

Korea Food Additives Code (2004). Maltogenic amylase Assay.

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

Lagrain, B., Leman, P., Goesaert, H., & Delcour, J. A. (2008). Impact of thermostable amylases during bread making on wheat bread crumb structure and texture. Food Research International, 41(8),819-827.

Liu M, Feng X, Ding Y, Zhao G, Liu H, Xian M. Metabolic engineering of *Escherichia coli* to improve recombinant protein production. Appl Microbiol Biotechnol. 2015;99(24):10367–10377.

Maksum I.P., Nabiel A., Ishmayana S, Soedjanaatmadja U. Safety Aspect of Recombinant Protein Produced by *Escherichia coli*: Toxin Evaluation with Strain and Genomic Approach. IntechOpen, pp.1-12; May 2020.

Marc J.E.C. van der Maarel, Bart van der Veen, Joost C.M. Uitdehaag, Hans Leemhuis, L. Dijkhuizen; Properties and applications of starch-converting enzymes of the alpha -amylase family; (2002); Journal of Biotechnology;94; 137-155

Mathesius CA, Barnett JF, Cressman RF, Ding J, Carpenter C, Ladics GS et al. 2009. Safety assessment of a modified acetolactate synthase protein (GM-HRA) used as a selectable marker in genetically modified soybeans. Regul Toxicol Pharmacol 55(3): 309-320.

Nilsson J, Jonasson P, Samuelsson E, Stahl S, Uhlen M. 1996. Integrated production of human insulin and its C-peptide. J Biotechnol. 48:241–250.

OECD Principles on Good Lab Practice (1998) Series on Principles of Good Laboratory Practice and Compliance Monitoring; 1-41

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; 1-16

OECD Test No. 423: Acute Oral toxicity - Acute Toxic Class Method (2001) OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing; 1-14

OECD Test No. 471: Bacterial Reverse Mutation Test (1997) OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing; 1-11

OECD Test No. 473: In Vitro Mammalian Chromosomal Aberration Test (1997) OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing; 1-10

Pariza MW & Johnson EA (2001). Evaluating the safety of microbial enzyme preparations used in food processing: Update for a new century. Regulatory Toxicology and Pharmacology, 33: 173-186.

Pence JW (1953) Panary Fermentation. Current Status of Problems. Journal of Agricultural and Food Chemistry, 1 (2), 157-161.

Quemada H, Zarka K, Pett W, Bothyma G, Felcher K, Mirendil H et al. 2010. Safety evaluation of the Cry11a1 protein found in the transgenic potato 'SpuntaG2'. J Am Soc Hortic Sci 135(4): 325-332.

Roifman CM, Mills GB, Chu M, Gelfand EW. 1985. Functional comparison of recombinant interleukin 2 (IL-2) with IL-2-containing preparations derived from cultured cells. Cell Immunol. 95(1):146-156.

Rosano GL, Ceccarelli EA. Recombinant protein expression in *Escherichia coli*: advances and challenges. Front Microbiol. 2014;5:172.

Safety Considerations for Biotechnology 1992, Organization for Economic Co-Operation and Development (OECD).

Studier W. F. Daegelen P., Lenski R. E., Maslov S., Kim J. F., Understanding the differences between genome sequences of *Escherichia coli* B Strains REL606 and BL21(DE3) and Comparison of the *E. coli* B and K-12 Genomes. J Mol Biol. 2009 Dec 11;394(4):653-80

Sweetness from Starch; FAO Corporate Document Repository (1996) http://www.fao.org/3/v9805e/V9805E00.htm

Szpirer CY, and Milinkovitch MC. 2005. Separate-component-stabilization system for protein and DNA production without the use of antibiotics. Biotechniques. 38(5): 775-781.

The Fresh Loaf Baker's Handbook http://www.thefreshloaf.com/handbook

Thomas K, Aalbers M, Bannon GA, Bartels M, et al. (2004); A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins; Regulatory Toxicology and Pharmacology; 39 (2004); 87–98.

USFDA GRN 624 GRAS Notification for D-allulose 3-epimerase from Arthrobacter globiformis M30 produced in *Escherichia coli*. Matsutani Chemical Industry Co. Ltd (2016)

USFDA GRN 746 GRAS Notification for Maltogenic amylase from *Geobacillus stearothermophilus* produced in *Bacillus subtilis*. AB Enzymes, Germany (2018).

USFDA GRN 751 GRAS Notification for Maltogenic alpha-amylase from *Bacillus stearothermophilus* produced in *Bacillus subtilis*. Novozymes North America (2018).

USFDA GRN 842 GRAS Notification for A Maltogenic a-amylase from *Geobacillus stearothermophilus* produced by *Saccharomyces cerevisiae*. Mascoma LLC (2019).

USFDA GRN 974 GRAS Notification for Maltogenic alpha-amylase enzyme preparation produced by *Bacillus subtilis*. AB Enzymes Inc. (2020).

USFDA GRN 975 GRAS Notification for Maltogenic alpha-amylase enzyme preparation produced by *Bacillus licheniformis* carrying the gene coding for maltogenic alpha-amylase from *Geobacillus stearothermophilus*. Novazymes NA (2020).

USP (2008). Unites States Pharmacopeia, the National Formulary USP31, NF26. Biological Tests and Assays: Antibiotics–Microbial Assay, pp91-96. Rockville, MD, USA.

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

Whitehead T. and Hespell B. 1989. Cloning and Expression in *Escherichia coli* of a Xylanase Gene from *Bacteroides ruminicola.* Applied and Environment Microbio. 55 (4): 893-896.

Xu F, Hinenoya A, Zeng X, Li XP, Guan Z, and Lin J; Critical Role of 3′-Downstream Region of pmrB in Polymyxin Resistance in Escherichia coli BL21(DE3); Microorganisms. 2021 Mar; 9(3): 655

7.2 Annex

Annex	Description	Reference	Pages
Annex A	Allergen declaration for maltogenic amylase	-	1
Annex B	Heavy Metals Analysis – TUV Nord Group	41400019, 71400440, 92100357	1-3
Annex C	Toxicity study summary of maltogenic amylase from GMM *E. coli* BLASC	-	1-13

advanced enzymes
Where ENZYME is Life

Manufacturing Site: Indore
Advanced Enzyme Technologies Ltd.
CIN: L24200MH1989PLC051018
Type 'B', Plot No. 5-13, Phase-1, Sector III, SEZ Indore,
Pithampur, District Dhar - 454 774, Madhya Pradesh, India.
Tel. / Fax: +91-7292-256 305
Email: info@advancedenzymes.com, Web.: www.advancedenzymes.com

Manufacturing Site: Nashik
Advanced Enzyme Technologies Ltd.
CIN: L24200MH1989PLC051018
Plot No. A-61/62, MIDC, Malegaon, Tal. Sinnar,
District Nashik - 422 113, Maharashtra, India.
Tel.: +91-997 010 0750 / +91-2551-230 044, Fax: +91-2551-230 816
Email: info@advancedenzymes.com, Web.: www.advancedenzymes.com

ALLERGEN INFORMATION

Product Name: MALTOGENIC AMYLASE

1. Cereals containing gluten, namely: wheat, rye, barley, oats, spelt, kamut or their hybridised strains, and products thereof, except[1]: (a) wheat based glucose syrups including dextrose ; (b) wheat based maltodextrins; (c) glucose syrups based on barley; (d) cereals used for making alcoholic distillates including ethyl alcohol of agricultural origin;	NO[2]
2. Crustaceans and products thereof;[1]	NO
3. Eggs and products thereof;[1]	NO
4. Fish and products thereof, except:[1] (a) fish gelatine used as carrier for vitamin or carotenoid preparations; (b) fish gelatine or Isinglass used as fining agent in beer and wine	NO
5. Peanuts and products thereof;[1]	NO
6. Soybeans and products thereof, except:[1] (a) fully refined soybean oil and fat; (b) natural mixed tocopherols (E306), natural D-alpha tocopherol, natural D-alpha tocopherol acetate, and natural D-alpha tocopherol succinate from soybean sources; (c) vegetable oils derived phytosterols and phytosterol esters from soybean sources; (d) plant stanol ester produced from vegetable oil sterols from soybean sources;	NO[2]
7. Milk and products thereof (including lactose), except:[1] (a) whey used for making alcoholic distillates including ethyl alcohol of agricultural origin; (b) lactitol;	NO[2]
8. Nuts[1], namely: almonds (*Amygdalus communis* L.), hazelnuts (*Corylus avellana*), walnuts (*Juglans regia*), cashews (*Anacardium occidentale*), pecan nuts (*Carya illinoinensis* (Wangenh.) K. Koch), Brazil nuts (*Bertholletia excelsa*), pistachio nuts (*Pistacia vera*), macadamia or Queensland nuts (*Macadamia ternifolia*), and products thereof, except for nuts used for making alcoholic distillates including ethyl alcohol of agricultural origin;	NO
9. Sesame seeds and products thereof;[1]	NO
10. Celery and products thereof;	NO
11. Mustard and products thereof;	NO
12. Sulphur dioxide and sulphites at concentrations of more than 10 mg/kg or 10 mg/litre in terms of the total SO_2 which are to be calculated for products as proposed ready for consumption or as reconstituted according to the instructions of the manufacturers;	NO
13. Lupin and products thereof;	NO
14. Molluscs and products thereof.	NO

1. USA, top 9 of the Food Allergy Safety, Treatment, Education, and Research (FASTER) Act, 2021.
2. Wheat products (Wheat bran, Wheat flour) Soy products (Soya flour, soy grit and soya peptone) and milk products (Casein and Lactose) are used in the manufacturing facility in which this product is made.

SR. MANAGER QUALITY
Advanced Enzyme Technologies Ltd
Date: 01/02/2022

ANNEX D

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

Test Report

Report No : TUV(I)/14/14-15/0041400019

Date : 08 Apr 2014

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

Reg No. 14/14-15

CA No. 0041400019

Date of sample receipt 01 Apr 2014

Date(s) of analysis 01 Apr 2014-07 Apr 2014

Sample Drawn by Customer

Sl No	Test Name	Result	Unit	LOQ/LOD	Test Method
	Sample Name : Maltogenic Amylase Batch No: 031434				**CA No : 0041400019**
	Heavy Metals				
1	Arsenic	<0.1	mg/kg	0.1	Based on AOAC 984.27 & 999.10,18th edition BY ICP-MS
2	Cadmium	<0.1	mg/kg	0.1	Based on AOAC 984.27 & 999.10,18th edition BY ICP-MS
3	Lead	<0.1	mg/kg	0.1	Based on AOAC 984.27 & 999.10,18th edition BY ICP-MS
4	Mercury	<0.025	mg/kg	0.025	Based on AOAC 984.27 & 999.10,18th edition BY ICP-MS

LOQ-Limit of Quantification
LOD-Limit of Detection

Verified by
Lalitkumar Thakur
Section Incharge – Instrumentation

Authorized by
V. K. Gupta
General Manager - Laboratory Operations

-- End of Report --

1. Test Results are based on & related only to the particular sample(s) tested.
2. This Report cannot be re-produced, except when in full, without the written permission from TUV India Pvt. Ltd.,
3. This Certificate reflects our findings at the time and place of testing.
4. Sample(s) will be retained by us for a period of one month for non-perishable items only. Perishable items will bedestroyed after completion of test.
5. This Report, in full or in part, shall not be used to make any misleading claims or for any legal purposes.

TUV INDIA
TÜV NORD GROUP

TUV INDIA PRIVATE LIMITED
TÜV India House,
Survey No. 42, 3/1 & 3/2,
Sus, Tal. Mulshi,
Dist. Pune - 411 021
CIN : U74140MH1989PTC052930
Tel. : 020 – 67900000 / 01
Email : pune@tuv-nord.com
Website : www.tuv-nord.com/in

Test Report

Report No : TUV(I)/2331/14-15/0071400440

Date : 14 Jul 2014

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

Reg No. : 2331/14-15

CA No. : 0071400440

Date of sample receipt : 09 Jul 2014

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

Sample Drawn by : Customer

Sl No	Test Name	Result	Unit	Test Method
	Sample Name : Maltogenic Amylase Batch No: 071404			**CA No : 0071400440**
	Heavy Metals			
1	Arsenic	<0.1	mg/kg	Based on AOAC 984.27 & 999.10,18th edition b y ICP-MS
2	Cadmium	<0.1	mg/kg	Based on AOAC 984.27 & 999.10,18th edition b y ICP-MS
3	Lead	<0.1	mg/kg	Based on AOAC 984.27 & 999.10,18th edition b y ICP-MS
4	Mercury	<0.025	mg/kg	Based on AOAC 984.27 & 999.10,18th edition b y ICP-MS

Verified by

Section Incharge – Instrumentation

Authorized by

Rehana Sheikh
Sr. Laboratory Analyst

-- End of Report --

1. Test Results are based on & related only to the particular sample(s) tested.
2. This Report cannot be re-produced, except when in full, without the written permission from TUV India Pvt. Ltd.,
3. This Certificate reflects our findings at the time and place of testing.
4. Sample(s) will be retained by us for a period of one month for non-perishable items only. Perishable items will bedestroyed after completion of test
5. This Report, in full or in part, shall not be used to make any misleading claims or for any legal purposes

TÜV NORD GROUP
TUV INDIA PRIVATE LIMITED
TUV India House,
Survey No: 42, 3/1 & 3/2,
Sus, Tal. Mulshi,
Dist. Pune - 411 021
CIN : U74140MH1989PTC052930
Tel. : 020 - 67900000 / 01
Toll free :1800-209-0902
Email : pune@tuv-nord.com
Website : www.tuv-nord.com/in

TEST REPORT

Report No : TUV(I)/6513/21-22/0092100357
Date : 09 Sep 2021

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

Reg No. : 6513/21-22

CA No. : 0092100357

Date of sample receipt : 03 Sep 2021

Date(s) of analysis : 03 Sep 2021 - 09 Sep 2021

Sample Drawn by : Customer

SINo	Test Name	Results	Unit	LOQ	Test Method
	Sample Name : MALTOGENIC AMYLASE Batch No.: 092101, Mfg. Date : September-2021 Discipline : Chemical			**CA No : 0092100357** Product Category : Food and Agriculture Product	
	Heavy Metals				
1	Arsenic	0.13	mg/kg	0.1	TUV/03/SOP/004 Based on AOAC 2015.01, 21 st Edition
2	Cadmium	< LOQ	mg/kg	0.1	TUV/03/SOP/004 Based on AOAC 2015.01, 21 st Edition
3	Lead	0.36	mg/kg	0.1	TUV/03/SOP/004 Based on AOAC 2015.01, 21 st Edition
4	Mercury	< LOQ	mg/kg	0.025	TUV/03/SOP/004 Based on AOAC 2015.01, 21 st Edition

LOQ-Limit of Quantification

Authorized by

Rehana Shaikh
Incharge – QA & Sr. Analyst

Note - This is Electronically Generated Report Copy

TÜV INDIA
TÜV NORD GROUP

TEST REPORT

Report No : TUV(I)/6513/21-22/0092100357
Date : 09 Sep 2021

Terms and Conditions

1. Test Results are based on & related only to the particular sample(s) tested.
2. This Report cannot be re-produced, except when in full, without the written permission from TUV India Pvt. Ltd.
3. This Certificate reflects our findings at the time and place of testing.
4. Sample(s) will be retained by us for a period of one month for non-perishable items only. Perishable items will be destroyed after completion of tests.
5. This Report, in full or in part, shall not be used to make any misleading claims or for any legal purposes.
6. All terms and conditions of our quotation on the basis of which this testing service has been provided are deemed to be fully accepted by the customer and are deemed to be in full force and effect.
7. This Report is exclusively for the use of the customer whose name and address is indicated above. No third party can derive rights against the company on the basis of this report. No third party has any right to raise any claims on the company.
8. For Biological and mycotoxin Analysis: Our analytical findings reflect the quality of the sample at the time of testing. No responsibility can be accepted for the possible consequences of further development of micro-organisms or mycotoxin which may depend upon storage, handling & weathers conditions which may influence the results at a later date/time respectively.
9. The laboratory tests are conducted by TUV India as per mutual agreement without any implied warranty of merchantability and fitness for any general or particular use. TUV India will not be liable for any claims for indirect, incidental or consequential damages including but not limited to loss of revenue, loss of profits and similar claims. TUV India will also not be liable under any strict liability, product liability or negligence. The total liability, scientifically and legally proven, if any, of TUV India, in any case, shall not exceed the invoiced amount for the services provided and paid for.
10. For Pesticide Residue analysis, reported value at LOQ level may vary within analytical variation of 50% considering Uncertainty of Measurement.

-- End of Report --

Toxicity Study Summary
of
Maltogenic amylase from GMM *E.coli* (strain BLASC)

ADVANCED ENZYME TECHNOLOGIES LTD.

Registered Office: 'A' Wing, Sun Magnetica, 5th Floor Accolade Galaxy,
L.I.C. Service road, Louiswadi, Thane – 400 604, India.
Tel.: 91-22-41703200 / 25838350,
Fax : 91-22-25835159
Website: http://www.advancedenzymes.com

Testing Facility : Intox Pvt. Ltd.
375, Urawade, Tal. Mulshi
Pune- 412108, India
Tel.:+91-20-66548700
Email:info@intoxlab.com

Regulatory compliance: The studies were conducted in compliance with Good Laboratory Principles (GLP) and OECD guidelines for testing of chemicals.

TABLE OF CONTENTS

Toxicity summary- Maltogenic amylase from GMM *E.coli* (strain BLASC)

1 TOXICITY SUMMARY ABSTRACT

The safety of the Maltogenic amylase from GMM *E.coli* (strain BLASC) is supported by the following series of toxicological studies. All studies were carried out in accordance with OECD guidelines and in compliance with the OECD principles of Good Laboratory Practice (GLP). The studies were performed at Intox Pvt Ltd (India) during May 2014 to January 2015

Test item	Batch No.	Enzyme Activity	Description	Regulatory compliance	Test Facility	Report No.	Year of study conducted
Maltogenic amylase	031434	144, 255 MAN U/g	Acute oral toxicity study in Wistar Rats	OECD 423 GLP	Intox Pvt. Ltd. India	14164	2014
Maltogenic amylase	031434	144, 255 MAN U/g	Repeated dose 90 day oral toxicity study by daily gavage in the rat followed by a 4 week recovery period.	OECD 408 GLP	Intox Pvt. Ltd. India	14165	2015
Maltogenic amylase	031434	144, 255 MAN U/g	Bacterial Reverse Mutation Assay	OECD 471 GLP	Intox Pvt. Ltd. India	14166	2014
Maltogenic amylase	031434	144, 255 MAN U/g	*In-vitro* Mammalian Chromosome Aberration test in Human Lymphocytes	OECD 473 GLP	Intox Pvt. Ltd. India	14167	2014

Following are the main conclusions of the studies in brief:

- Acute Oral Toxicity study (OECD 423): Maltogenic amylase batch 031434 was tested in Wistar rats by Acute Toxicity class method as described by OECD 423 guideline. The product found to be safe and classified as GHS category 5 or Unclassified for obligatory labelling requirements. LD_{50} cut-off value being 5000 mg/kg body weight.
- Repeated 90-days oral toxicity (OECD 408): Based on 90 days repeated dose oral toxicity study in Wistar rats, the No-Observed-Adverse-Effect-Level (NOAEL) of Maltogenic amylase was found to be equal to or greater than 1000 mg/kg body weight.
- Bacterial Reverse Mutation Assay (OECD 471): Maltogenic amylase was evaluated in Ames/Salmonella Pre-incubation assay. The product found to be non-mutagenic in Salmonella strains TA1635, TA97a, TA98, TA100 & TA102
- *In vitro* chromosomal aberration test in human lymphocytes (OECD 473): The result indicated that Maltogenic amylase did not induce significant chromosome aberrations in cultured mammalian cell, either in presence or in absence of metabolic activation system. Therefore, considered as non-clastogenic in cultured mammalian cells, peripheral blood lymphocyte.

2 TEST SUBSTANCE

The toxicity study batch of Maltogenic amylase was produced according to the procedure used for commercial production. The tox-batch specification and other details are presented below:

Sample : Maltogenic amylase from GMM *E.coli* (strain BLASC):

Batch no. : 031434

Activity : 144,255 MAN U/g

TOS : 84.72%

Batch No.	**031434**
Ash (%)	8.86
Water (%)	7.35
TOS (%)*	83.79
Activity (U/g MAN)	144,255
MAN U/mg TOS	172.16
Protein (%)	45.18
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

3 ASSESSMENT OF SYSTEMIC TOXICITY

ACUTE ORAL TOXICITY STUDY

Acute oral toxicity study of Maltogenic amylase in Wistar rats was performed as per OECD Guideline for Testing of Chemicals, Section 4, No. 423 - Acute Oral Toxicity - Acute Toxic Class Method, adopted 17 December 2001.

The method uses pre-defined doses and the results allow a substance to be ranked and classified according to the Globally Harmonised System (GHS) for classification of chemicals, which cause acute toxicity.

Method:

In this study, single oral administration of test article was made to groups of three female Wistar rats in step-wise manner to assess its acute toxicity. The test article was suspended in analytical grade water to obtain concentration of 200 mg/ml.

In step 1 of the study, test article was administered to three rats, at the dose of 2000 mg/kg body weight. Test article did not induce any mortality and abnormal clinical signs in treated rats.

In step 2, when further tested on three rats at the dose of 2000 mg/kg body weight, test article did not induce any mortality and abnormal clinical signs in treated rats.

Results/Observations:

The body weight gain by treated rats was not affected during observation period. No gross pathological alterations were encountered in the rats when sacrificed at termination of the study.

Conclusion:

Based on these results, and according to the 'Globally Harmonised System' (GHS) for classification of chemicals which cause acute toxicity, OECD series on testing and assessment, Number 33; Harmonised Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures [ENV/JM/MONO(2001)6], the test article, Maltogenic amylase has to be classified in GHS Category 5 or Unclassified for the obligatory labelling requirement for oral toxicity, the corresponding LD50 cut-off value being 5000 mg/kg body weight.

Toxicity summary- Maltogenic amylase from GMM *E.coli* (strain BLASC)

REPEATED DOSE 90-DAYS ORAL TOXICITY STUDY

The aim of this study was to investigate the clinical, haematological and histological toxicity potential of Maltogenic amylase from GMM *E.coli (strain BLASC)* (Batch no. 031434), 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 Maltogenic amylase when administrated daily for 90 days in the Sprague Dawley rats.

Method:

Groups of ten male and ten female Wistar rats were administered test article Maltogenic amylase by oral gavage daily at the doses of 250, 500 and 1000 mg/kg of body weight for 90 days and were sacrificed on day 91 to evaluate its toxicity. A concurrent control group of ten males and ten females receiving the vehicle, i.e. analytical grade water at 5 ml/kg was also maintained for 90 days. Additionally, groups of five rats per sex which had received the vehicle at 5 ml/kg and the test article at the high dose level, i.e. 1000 mg/kg body weight, were further observed for a period of 28 days following the 90 days treatment, for assessment of reversibility, persistence or delayed occurrence of toxicity.

The rats were examined daily for signs of toxicity, morbidity and mortality. They were subjected to detailed clinical examination before initiation of the study and weekly thereafter during the treatment period, recovery period and at termination. Ophthalmoscopic examination was conducted on control and high dose group animals before initiation of the study and at termination of treatment. In the thirteenth week of treatment, animals were additionally examined for assessment of sensory reactivity, assessment of grip strength and motor activity. Body weight and food consumption were recorded weekly. Laboratory investigations were performed on blood and urine at termination of the treatment and at the end of recovery period. All animals sacrificed terminally were subjected to a detailed necropsy and weights of kidneys, liver, adrenals, testes, epididymides, uterus, thymus, spleen, brain, ovaries and heart were recorded. Histopathological evaluation was performed on all tissues [brain, spinal cord, eyes, pituitary, thyroid, parathyroid, spleen, thymus, adrenals, pancreas, trachea, lungs, heart, aorta, oesophagus, stomach, duodenum, jejunum, terminal ileum (with peyer's patch), colon, rectum, liver, kidneys, urinary bladder, prostate, seminal vesicle, epididymides, testes, ovaries, utérus with cervix, skin, vagina, sciatic nerve, bone marrow (smear), mammary gland (females), mesenteric lymph node, axillary lymph node and salivary glands] in all rats from the control and high dose groups.

Toxicity summary- Maltogenic amylase from GMM *E.coli* (strain BLASC)

Results/Observations:

There was no incidence of treatment related mortality in rats treated with the test article at any of the dose levels. The test article did not induce any remarkable or treatment related clinical abnormalities in rats treated at and up to the dose of 1000 mg/kg body weight. No mortality or abnormal clinical signs were observed in the vehicle control animals. Ophthalmological examination did not reveal any treatment related ocular abnormalities. Also, the observations on sensory reactivity, grip strength and motor activity conducted in the thirteenth week of treatment did not reveal any neurotoxic potential of the test article.

Body weight gain was not affected in male and female rats treated at and up to the dose of 1000 mg/kg and were found to be comparable to that by the control rats throughout the treatment period and also during the recovery period. The test article did not have any adverse effect on the average daily food consumption by the male and female rats treated at any of the dose levels.

The hematological parameters of hemoglobin, packed cell volume, total RBC count, total and differential WBC counts, RBC indices, platelet count, activated partial thromboplastin time and prothrombin time of male and female rats treated with the test article were found to be comparable to those of the vehicle control animals at termination of the treatment and also at the end of the recovery period.

The test article did not alter the plasma levels of total protein, albumin, globulin, alanine aminotransferase, asparate aminotransferase, alkaline phosphatase, glucose, creatinine, calcium, total cholesterol, phosphorous, total bilirubin, urea nitrogen, urea, sodium, potassium and triglycerides in male and female rats. The data on urinalysis indicated no adverse effect due to the treatment.

The values of absolute and relative weights of kidneys, liver, adrenals, testes, epididymides, uterus, thymus, spleen, brain, ovaries and heart of male and female rats treated with test article, were found to be comparable with those of the control rats at the end of treatment period and also at the end of the recovery period.

No treatment related gross pathological changes were noted. Histopathological examination was performed on tissues of the control and high dose group animals, where the changes in the high dose group were incidental or comparable to the control group or unrelated to treatment.

Conclusion:

Based on the findings of this study it was concluded that, the No-Observed-Adverse-Effect-Level (NOAEL) of Maltogenic amylase in Wistar rats, following oral administration for 90 days was found to be equal to or greater than 1000 mg/kg body weight.

4 ASSESSMENT OF GENOTOXICITY

BACTERIAL REVERSE MUTATION ASSAY (AMES TEST)

The aim of this *in-vitro* study was to detect the possible presence of compounds or contaminants in the test substance, Maltogenic amylase from GMM *E.coli* (strain BLASC) (Batch no. 031434). 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 Maltogenic amylase from GMM *E.coli* (strain BLASC) (Batch no. 031434) for its ability to induce gene mutations, the bacterial reverse mutation test was conducted using *Salmonella typhimurium* tester strains viz., TA100, TA102, TA97a, TA98 and TA1535.

Method:

Initially, the preliminary tests were conducted to assess the solubility / precipitation and cytotoxicity of Maltogenic amylase, the tester strains were exposed to the test article in triplicate cultures at the doses of 5000 µg, 1500 µg, 500 µg, 150 µg and 50 µg/plate in presence and in absence of metabolic activation system. Liver S_9, induced in Wistar rats by phenobarbitone with β - naphthoflavone, was used for this purpose.

Analytical grade water was used as a vehicle and appropriate positive controls (Methyl methane sulphonate, Sodium azide, 4-Nitroquinolene-N-Oxide, ICR-191 for without metabolic activation and 2-Aminofluorene, 2-Aminoanthracene and Danthron for with metabolic activation) were tested simultaneously. The exposed bacteria were plated onto minimal glucose agar medium supplemented with L-histidine. The plates were incubated at 37°C for about 67 hours after which the histidine revertant colonies were counted and their frequency was compared with that in the vehicle control group. Concurrent positive control groups were also included in the experiment, as specified by the test guideline.

Results/Observations:

Results of this test indicated that the frequencies of histidine revertant colonies at all concentrations of Maltogenic amylase in strains TA1535, TA97a, TA98, TA100 and TA102, in presence and absence of a metabolic activation system, were comparable to those observed in the vehicle control group. As per the criteria employed for evaluation of mutagenic potential this observation confirmed by repetition of the experiments.

Toxicity summary- Maltogenic amylase from GMM *E.coli* (strain BLASC)

Table 1: Summary of Bacterial reverse mutation test

Treatment	Concentration		TA1535		TA97a		TA98		TA100		TA102	
	(µg/plate)	S9	Mean	Multiples of Vehicle control	Mean	Multiples of Vehicle control	Mean	Multiples of Vehicle control	Mean	Multiples of Vehicle control	Mean	Multiples of Vehicle control
Maltogenic amylase	5000	-	13.67	1.08	109.33	1.00	30.67	1.14	138.67	1.12	242.67	0.89
		+	18.00	1.23	114.00	0.96	25.67	0.78	202.00	0.89	262.67	0.97
	1500	-	13.00	1.03	113.33	1.04	29.33	1.09	140.67	1.14	268.00	0.99
		+	16.33	1.11	123.33	1.03	36.33	1.10	132.00	0.87	257.33	0.95
	500	-	15.00	1.18	116.00	1.06	23.33	0.86	126.00	1.02	254.67	0.94
		+	17.67	1.20	125.33	1.05	33.67	1.02	146.00	0.96	264.00	0.98
	150	-	13.33	1.05	120.00	1.10	22.67	0.84	121.33	0.98	272.67	1.00
		+	14.67	1.00	117.33	0.98	41.00	1.24	129.33	0.85	234.67	0.87
	50	-	14.67	1.16	110.00	1.01	28.67	1.06	124.00	1.01	275.33	1.01
		+	15.00	1.02	127.33	1.07	34.33	1.04	141.33	0.93	269.33	1.00
Vehicle Control												
Analytical grade water	100 µl	-	12.67	1.00	109.33	1.00	27.00	1.00	123.33	1.00	271.33	1.00
		+	14.67	1.00	119.33	1.00	33.00	1.00	152.00	1.00	270.67	1.00
Positive Controls												
Sodium azide	2	-	624.00	49.26								
ICR 191	1	-			886.67	8.11						
4-Nitroquinoline-N-oxide	0.5	-					508.00	18.81				
3-Methylmethane Sulphonate	1 µl	-							1592.00	12.91	1800.00	6.63
2-Aminoanthracene	10	+	504.00	34.36								
2-Aminofluorene	20	+			869.33	7.28	507.33	15.37	1296.00	8.53		
Danthron	30	+									1482.67	5.48

S9: - Without S9 + With S9

Plate counts for the spontaneous histidine revertant colonies in the vehicle control groups were found to be within the frequency, compared well with range reported in the published literature. There was no any dose dependent or 2-fold (3-fold for TA1535) increase in number of revertant colonies as compared to vehicle control group. The number of revertant colonies as in the positive controls increased by 5.48 to 49.26 fold under identical conditions, demonstrated sensitivity of the assay both in presence and absence of metabolic activation.

Conclusions:

Based on the results it was concluded that Maltogenic amylase tested at and up to 5000.00 µg /plate concentration did not induce any mutations in the *Salmonella* strains (TA1535, TA97a, TA98, TA100 and TA102) used in the study and is not mutagenic in this Bacterial Reverse Mutation Test.

Toxicity summary- Maltogenic amylase from GMM *E.coli* (strain BLASC)

IN VITRO CHROMOSOMAL ABERRATION TEST IN HUMAN LYMPHOCYTES

The aim of this *in vitro* study was to detect the possible presence of compounds or contaminants in the test substance, Maltogenic amylase (batch no. 031434), 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 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.

The study was conducted to evaluate the clastogenic potential of Maltogenic amylase from GMM *E.coli (strain BLASC)* (Batch no. 031434), as judged by the ability to induce chromosomal aberrations in an *in* cultured mammalian somatic cells, viz. human peripheral blood lymphocytes.

Method:

Testing was conducted in three separate experiments, two in absence of supplementary metabolic activation system (S_9) and one in presence. Liver S_9, induced in rats by phenobarbitone with β-napthoflavone, was used for this purpose. Three exposure levels were employed along with concurrent vehicle control and positive control groups.

Following preliminary solubility / precipitation and cytotoxicity studies, cultures of human peripheral blood lymphocytes were exposed to the test article formulated in analytical grade water (vehicle) at concentrations of 5000 µg/ml, 1500 µg/ml and 500 µg/ml in the absence (Expt.1) and presence (Expt.3) of S_9 for an exposure of 3 hours and (Expt.2) in the absence of S_9 for an exposure period of 24 hours.

All cell cultures were treated with colchicine (0.5µg/ml) at 2-3 hours prior to harvesting. Each culture was harvested at a sampling time equivalent to about 1.5 cell cycle length and chromosomal preparations were made and stained with 5% Giemsa (v/v). Two hundred well spread metaphases were evaluated microscopically for structural aberrations per test concentration. The incidence of chromosome aberrations in the treatment group were compared with that in the vehicle control groups. The entire study was carried out in duplicates cultures at each concentration.

Results/Observations:

When compared to the vehicle control group, no significant and concentration related increase was observed in the incidence of structural chromosome aberrations in the human peripheral blood lymphocytes, exposed at any of the concentrations of Maltogenic amylase, either in presence or in absence of metabolic activation system.

Toxicity summary- Maltogenic amylase from GMM *E.coli* (strain BLASC)

Sensitivity of the test system and activity of S_9 mix were demonstrated in the positive control groups. The positive controls, directly acting mutagen Methyl methane sulphonate and promutagen Cyclophosphamide, induced significant increase in chromosome aberration frequencies over the concurrent controls, which validated the test method.

Table 2: Summary of Chromosomal aberrations test (3h) without metabolic activation (S_9)

Test / Control Article	Dose µg/ml	No. of Cells Analysed	No. of Aberrated Cells#		No. of Aberrations												Aberrations Excluding Gaps#		
			Including Gaps	Excluding Gaps	Gaps		Breaks		AF	DEL	DC	RC	Other		Total	Total	Nos. per Cell	% Cells with Aberrations
					CS	CT	CS	CT					PP	EDR				
Vehicle Control Analytical grade water	0	200	4	3	0	1	1	0	0	0	2	0	1	0	5	3	0.02	1.50
Positive Control MMS	30	100	30	28	1	1	5	8	3	1	12	1	0	0	32	30	0.3	28.00*
Maltogenic Amylase	500	200	9	6	1	2	0	1	0	0	5	0	1	0	10	6	0.03	3.00
	1500	200	10	8	1	1	1	2	0	1	4	0	0	0	10	8	0.04	4.00
	5000	200	8	6	1	1	2	3	0	0	1	0	1	2	11	6	0.03	3.00

Numerical aberrations have also been excluded. * $p > 0.05$

Table 3: Summary of Chromosomal aberrations test (3h) with metabolic activation (S_9)

Test / Control Article	Dose µg/ml	No. of Cells Analysed	No. of Aberrated Cells#		No. of Aberrations											Aberrations Excluding Gaps#		
			Including Gaps	Excluding Gaps	Gaps		Breaks		AF	DEL	DC	PV	Other		Total	Total	Nos. per Cell	% Cells with Aberrations
					CS	CT	CS	CT					PP	EDR				
Vehicle Control Analytical grade water	0	200	5	4	0	1	1	1	0	1	1	0	0	0	5	4	0.02	2.00
Positive Control CPM	60	100	43	41	4	0	13	15	1	1	19	0	0	0	53	49	0.49	41.00*
Maltogenic Amylase	500	200	9	7	0	2	1	2	1	0	3	0	0	0	9	7	0.04	3.50
	1500	200	7	6	0	1	0	1	1	1	3	0	1	0	8	6	0.03	3.00
	5000	200	9	8	0	1	3	4	1	0	1	0	0	0	10	9	0.05	4.00

Numerical aberrations have also been excluded. * $p > 0.05$

Toxicity summary- Maltogenic amylase from GMM *E.coli* (strain BLASC)

Table 4: Summary of Chromosomal aberrations test (24h) without metabolic activation (S_9)

Test / Control Article	Dose µg/ml	No. of Cells Analysed	No. of Aberrated Cells #		No. of Aberrations												Aberrations Excluding Gaps #		
			Including Gaps	Excluding Gaps	Gaps		Breaks		AF	DEL	DC	RC	Other		Total	Total	Nos. per Cell	% Cells with Aberrations
					CS	CT	CS	CT					PP	EDR				
Vehicle Control																		
Analytical grade water	0	200	5	3	1	1	1	1	0	0	1	0	0	0	5	3	0.02	1.50
Positive Control MMS	30	100	33	29	1	3	4	13	1	0	12	0	0	0	34	30	0.3	29.00*
	500	200	12	7	1	4	1	2	0	1	3	0	0	0	12	7	0.04	3.50
Maltogenic Amylase	1500	200	6	4	2	0	0	0	0	0	4	0	1	0	7	4	0.02	2.00
	5000	200	12	8	3	1	2	2	1	0	3	0	0	0	12	8	0.04	4.00

Numerical aberrations have also been excluded. * $p > 0.05$

Assessment of mitotic indices made during the preliminary cytotoxicity test and the main study indicated that Maltogenic amylase did not exhibit severe cytotoxic effect on the cultured human lymphocytes at the maximum test concentration of 5000 µg/ml during 3 hours, 24 hours of exposure in absence of metabolic activation system and 3 hours of exposure in presence of metabolic activation system.

Conclusion:

Based on these results it is concluded that Maltogenic amylase 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.

5 REFERENCES:

1. OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring, Number 1, "OECD Principles on Good Laboratory Practice" ENV/MC/CHEM(98)17 (as revised in 1997), OECD 1998.

2. OECD Guideline No. 423; 'Acute Oral Toxicity – Acute Toxic Class Method'. The Organization for Economic Co-operation and development (OECD) guidelines for the Testing of Chemicals, adopted on 17 December 2001

3. OECD Guideline No. 408; 'Repeated Dose 90-Day Oral Toxicity Study In Rodents' The Organization for Economic Co-operation and development (OECD) guidelines for the Testing of Chemicals, adopted on 21 September 1998

4. OECD Guideline No. 471; 'Bacterial Reverse Mutation Test' The Organization for Economic Co-operation and development (OECD) guidelines for the Testing of Chemicals, adopted on 21 July 1997

5. OECD Guideline No. 473; 'In vitro Mammalian Chromosome Aberration Test', The Organization for Economic Co-operation and development (OECD) guidelines for the Testing of Chemicals, adopted on 21 July 1997

SCIENTIFIC OPINION

EFSA Journal

ADOPTED: 23 January 2018

doi: 10.2903/j.efsa.2018.5171

Safety evaluation of the food enzyme maltogenic amylase from a genetically modified *Bacillus subtilis* (strain NZYM-SM)

EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF), Vittorio Silano, Claudia Bolognesi, Laurence Castle, Kevin Chipman, Jean-Pierre Cravedi, Paul Fowler, Roland Franz, Konrad Grob, Rainer Gürtler, Trine Husøy, Sirpa Kärenlampi, Wim Mennes, Maria Rosaria Milana, Karla Pfaff, Gilles Riviere, Jannavi Srinivasan, Maria de Fatima Tavares Poças, Christina Tlustos, Detlef Wölfle, Holger Zorn, Andrew Chesson, Boet Glandorf, Lieve Herman, Klaus-Dieter Jany, Francesca Marcon, Andre Penninks, Andrew Smith, Henk van Loveren, Davor Zeljezic, Margarita Aguilera-Gomez, Magdalena Andryszkiewicz, Davide Arcella, Natalia Kovalkovicova, Yi Liu and Karl-Heinz Engel

Abstract

The food enzyme considered is a maltogenic amylase (glucan 1,4-α-maltohydrolase; EC 3.2.1.133) produced with the genetically modified *Bacillus subtilis* strain NZYM-SM by Novozymes A/S. The food enzyme contains neither the production organism nor recombinant DNA. The maltogenic amylase is intended for use in baking processes and starch processing for glucose syrups production. Based on the maximum use levels recommended for the food processes and individual consumption data from the EFSA Comprehensive European Food Consumption Database, dietary exposure to the food enzyme–Total Organic Solids (TOS) was estimated to be up to 0.168 mg TOS/kg body weight (bw) per day in European populations. The food enzyme did not induce gene mutations in bacteria or chromosomal aberrations in human lymphocytes. The subchronic toxicity was assessed by means of a repeated dose 90-day oral toxicity study in rodents. A no observed adverse effect level (NOAEL) was derived (320 mg TOS/kg bw per day), which, compared with the dietary exposure, results in a sufficiently high margin of exposure. The allergenicity was evaluated by searching for similarity of the amino acid sequence to those of known allergens. Three matches to occupational respiratory allergens were found, however, the Panel considered that there are no indications for food allergic reactions to the food enzyme. Based on the genetic modifications performed, the manufacturing process, the compositional and biochemical data provided, the dietary exposure assessment, the findings in the toxicological studies and allergenicity assessment, the Panel concluded that the food enzyme maltogenic amylase from *Bacillus subtilis* strain NZYM-SM does not give rise to safety concerns under the intended conditions of use.

Keywords: food enzyme, glucan 1, 4-α-maltohydrolase, EC 3.2.1.133, maltogenic amylase, *Bacillus subtilis*, genetically modified microorganism

Requestor: European Commission

Question number: EFSA-Q-2015-00096

Correspondence: fip@efsa.europa.eu

Panel members: Claudia Bolognesi, Laurence Castle, Kevin Chipman, Jean-Pierre Cravedi, Karl-Heinz Engel, Paul Fowler, Roland Franz, Konrad Grob, Rainer Gürtler, Trine Husøy, Sirpa Kärenlampi, Wim Mennes, Maria Rosaria Milana, Karla Pfaff, Gilles Riviere, Vittorio Silano, Jannavi Srinivasan, Maria de Fatima Tavares Poças, Christina Tlustos, Detlef Wölfle and Holger Zorn.

Note: The full opinion will be published in accordance with Article 12 of Regulation (EC) No 1331/2008 once the decision on confidentiality will be received from the European Commission.

Acknowledgements: The Panel wishes to thank Ana Gomes and Joaquim Maia for the support provided to this scientific output.

Suggested citation: EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (EFSA CEF Panel), Silano V, Bolognesi C, Castle L, Chipman K, Cravedi J-P, Fowler P, Franz R, Grob K, Gürtler R, Husøy T, Kärenlampi S, Mennes W, Milana MR, Pfaff K, Riviere G, Srinivasan J, Tavares Poças MF, Tlustos C, Wölfle D, Zorn H, Chesson A, Glandorf B, Herman L, Jany K-D, Marcon F, Penninks A, Smith A, van Loveren H, Zeljezic D, Aguilera-Gomez M, Andryszkiewicz M, Arcella D, Kovalkovicova N, Liu Y and Engel K-H, 2018. Scientific Opinion on the safety evaluation of the food enzyme maltogenic amylase from a genetically modified *Bacillus subtilis* (strain NZYM-SM). EFSA Journal 2018;16(5):5171, 21 pp. https://doi.org/10.2903/j.efsa.2018.5171

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

Table of contents

1. Introduction

Article 3 of the Regulation (EC) No 1332/2008[1] provides definitions for 'food enzyme' and 'food enzyme preparation'.

'Food enzyme' means a product obtained from plants, animals or microorganisms or products thereof including a product obtained by a fermentation process using microorganisms: (i) containing one or more enzymes capable of catalysing a specific biochemical reaction; and (ii) added to food for a technological purpose at any stage of the manufacturing, processing, preparation, treatment, packaging, transport or storage of foods.

'Food enzyme preparation' means a formulation consisting of one or more food enzymes in which substances such as food additives and/or other food ingredients are incorporated to facilitate their storage, sale, standardisation, dilution or dissolution.

Before January 2009, food enzymes other than those used as food additives were not regulated or were regulated as processing aids under the legislation of the Member States. On 20 January 2009, Regulation (EC) No 1332/2008 on food enzymes came into force. This Regulation applies to enzymes that are added to food to perform a technological function in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food, including enzymes used as processing aids. Regulation (EC) No 1331/2008 established European Union (EU) procedures for the safety assessment and the authorisation procedure of food additives, food enzymes and food flavourings. The use of a food enzyme shall be authorised only if it is demonstrated that:

i) it does not pose a safety concern to the health of the consumer at the level of use proposed;
ii) there is a reasonable technological need;
iii) its use does not mislead the consumer.

All food enzymes currently on the EU market and intended to remain on that market, as well as all new food enzymes, shall be subjected to a safety evaluation by the European Food Safety Authority (EFSA) and an approval via a Union list.

The 'Guidance on submission of a dossier on a food enzyme for evaluation' (EFSA, 2009a) lays down the administrative, technical and toxicological data required.

1.1. Background and Terms of Reference as provided by the requestor

1.1.1. Background as provided by the European Commission

Only food enzymes included in the Union list may be placed on the market as such and used in foods, in accordance with the specifications and conditions of use provided for in Article 7 (2) of Regulation (EC) No 1332/2008 on food enzymes.

Five applications have been introduced by the companies 'Novozymes A/S', 'AB Enzymes GmbH'. 'Ajinomoto Europe SAS' and 'Nagase (Europa) GmbH' for the authorisation of the food enzymes Beta-galactosidase from a genetically modified strain of *Bacillus licheniformis* (strain NZYM-BT), Mannan endo-1,4-beta-mannosidase (β-mannanase) from a genetically modified strain of *Trichoderma reesei* (strain RF6232), Transglutaminase from *Streptoverticillium mobaraense* (strain S-8112), Maltogenic amylase from a genetically modified strain of *Bacillus subtilis* (strain NZYM-SM) and Glucanase from *Streptomyces violaceoruber* (strain pGlu).

Following the requirements of Article 12.1 of Commission Regulation (EU) No 234/2011[2] implementing Regulation (EC) No 1331/2008[3], the Commission has verified that the five applications fall within the scope of the food enzyme Regulation and contains all the elements required under Chapter II of that Regulation.

[1] Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on Food Enzymes and Amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97. OJ L 354, 31.12.2008, p. 7–15.

[2] Commission Regulation (EU) No 234/2011 of 10 March 2011 implementing Regulation (EC) No 1331/2008 of the European Parliament and of the Council establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 64, 11.3.2011, p. 15–24.

[3] Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 354, 31.12.2008, p. 1–6.

1.1.2. Terms of Reference

The European Commission requests the European Food Safety Authority to carry out the safety assessments on the food enzymes Beta-galactosidase from a genetically modified strain of *Bacillus licheniformis* (strain NZYM-BT), Mannan endo-1,4-beta-mannosidase (β-mannanase) from a genetically modified strain of *Trichoderma reesei* (strain RF6232), Transglutaminase from *Streptoverticillium mobaraense* (strain S-8112), Maltogenic amylase from a genetically modified strain of *Bacillus subtilis* (strain NZYM-SM) and Glucanase from *Streptomyces violaceoruber* (strain pGlu) in accordance with the article 17.3 of Regulation (EC) No 1332/2008 on food enzymes.

1.2. Interpretation of the Terms of Reference

The present scientific opinion addresses the European Commission request to carry out the safety assessment of the food enzyme Maltogenic amylase from a genetically modified strain of *Bacillus subtilis* (strain NZYM-SM).

1.3. Information on existing authorisations and evaluations

The applicant reports that the Danish and French authorities have evaluated and authorised the use of the food enzyme from a genetically modified *Bacillus subtilis* strain NZYM-SM in starch processing, baking processes and maltose syrup production.

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier supporting the application for authorisation of the food enzyme maltogenic amylase from a genetically modified strain of *B. subtilis* (strain NZYM-SM).

2.2. Methodologies

The assessment was conducted in line with the principles described in the EFSA 'Guidance on transparency in the scientific aspects of risk assessment' (EFSA, 2009b) and following the relevant existing Guidances from the EFSA Scientific Committee.

The current 'Guidance on the submission of a dossier for safety evaluation of a food enzyme' (EFSA CEF panel, 2009) has been followed for the evaluation of this application with the exception of the exposure assessment, which was carried out in accordance to the methodology described in the CEF Panel statement on the exposure assessment of food enzymes (EFSA CEF Panel, 2016).

3. Assessment

3.1. Technical data

3.1.1. Identity of the food enzyme

IUBMB nomenclature: Glucan 1,4-α-maltohydrolase
Systematic name: 4-α-D-glucan α-maltohydrolase
Synonyms: Maltogenase
IUBMB No: EC 3.2.1.133
CAS No: 160611-47-2.

3.1.2. Chemical parameters

The maltogenic amylase produced with the genetically modified *Bacillus subtilis* strain NZYM-SM consists of a single polypeptide of 686 amino acids. The molecular mass, derived from the amino acid sequence, was calculated to be 75.2 kDa. The sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel analysis consistently showed one major protein band in all batches, migrated slightly below 66 kDa reference protein.

The food enzyme was tested for other enzyme activities, i.e. lipase, protease and glucoamylase, which were below the limits of quantification (LOQ) of the applied assays, except for one commercial

batch with a very low amount of glucoamylase activity. No other enzyme activities relevant to the intended uses were reported by the applicant.

Data on the chemical parameters of the food enzyme were provided for four food enzyme batches, three batches used for commercialisation and one batch used for the toxicological tests (Table 1). The average Total Organic Solids (TOS) content of the three food enzyme batches used for commercialisation was 4.4%; the values ranged from 2.9% to 5.3% (Table 1).

The average enzyme activity/TOS ratio of the three food enzyme batches used for commercialisation was 330 Maltogenic Amylase Novo Units MANU/mg TOS; the values ranged from 273 to 362 MANU/mg TOS (Table 1). The average activity/TOS ratio of 330 MANU/mg TOS was used for subsequent calculations.

Table 1: Compositional data provided for the food enzyme

Parameter	Unit	Batch			
		1	2	3	4[(a)]
Maltogenic amylase activity	MANU/g batch[(b)]	19,200	10,300	13,400	8,600
Protein	%	4.9	2.9	4.7	ND
Ash	%	1.0	0.8	1.1	4.8
Water	%	93.7	96.3	94.0	86.1
Total organic solids (TOS)[(c)]	%	5.3	2.9	4.9	9.1
Activity/mg TOS	MANU/mg TOS	362	355	273	94.5

ND: not determined.
(a): Batch used for the toxicological studies.
(b): MANU: Maltogenic Amylase Novo Units (see Section 3.1.3).
(c): TOS calculated as 100% - % water - % ash.

The food enzyme complies with the specification for lead (not more than 5 mg/kg) as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006). In addition, the levels of arsenic, cadmium and mercury were below the limits of detection of the employed methodologies.

No antimicrobial activity was detected in any of these batches (FAO/WHO 2006).

The food enzyme complies with the microbiological criteria as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006), which stipulate that *Escherichia coli* and *Salmonella* species are absent in 25 g of sample and total coliforms should not exceed 30 colony forming units (CFU) per gram.

The applicant has provided information on the identity of the antifoam agents used. Taking into account the nature and properties of the antifoam agents, the manufacturing process and the quality assurance system implemented by the applicant, the Panel considers their use as of no safety concern.

The Panel considered the compositional data provided for the food enzyme as sufficient.

3.1.3. Properties of the food enzyme

The maltogenic amylase catalyses the hydrolysis of (1→4)-α-D-glucosidic linkages in amylose, amylopectin and related glucose polymers, liberating maltose units from the non-reducing end of the polymer chain.

The enzymatic activity is determined on the basis of an in-house method using a maltotriose standard and expressed in Maltogenic Amylase Novo Units/g (MANU/g). One MANU is defined as the amount of enzyme that produces 1 μmol glucose per minute using maltotriose as substrate under the defined assay conditions (reaction conditions: pH = 5.0, T = 37°C, incubation time 30 min). The enzymatic hydrolysis of maltotriose results in the release of glucose, which is determined quantitatively using a glucose hexokinase assay.

The food enzyme has been characterised regarding its activity depending on temperature and pH. The temperature profile of the food enzyme was measured from 40°C to 100°C. The maltogenic amylase is active at temperatures below 90°C with an optimum between 60°C and 70°C at pH 5.5. The activity is completely lost after incubation of the enzyme for 30 min at 90°C. The pH optimum is around pH 5 at 30°C.

3.1.4. Information on the source material

3.1.4.1. Information relating to the genetically modified microorganism

The maltogenic amylase production strain *Bacillus subtilis* NZYM-SM is deposited in the [redacted] with the deposit number [redacted].

3.1.4.2. Characteristics of the parental and recipient microorganism

The parental microorganism is the bacterium *B. subtilis*, strain [redacted] ([redacted]).

The identity of the parental strain has been confirmed by whole genome sequencing ([redacted]). The parental strain has been tested for the absence of cytotoxicity in VERO cells. An intermediate strain has been tested both in CHO-K1 (Pedersen et al., 2002) and in VERO cells. Both proved negative.

The recipient strain, *B. subtilis* [redacted], has been developed from the parental strain [redacted]

3.1.4.3. Characteristics of the donor organisms

[redacted]

3.1.4.4. Description of the genetic modification process

The production strain NZYM-SM was developed from the recipient strain [redacted]

[redacted]

3.1.4.5. Safety aspects of the genetic modification

[redacted]

3.1.5. Manufacturing process

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004[4] and in accordance with current Good Manufacturing Practice (GMP).

The food enzyme is produced by a pure culture in a contained, submerged, fed-batch fermentation system with conventional process controls in place. The identity and purity of the culture are checked at each transfer step from frozen vials to the end of fermentation.

The food enzyme produced is recovered from the fermentation broth after biomass separation using filter press filtration. Further purification and concentration involve a series of filtration steps, including ultrafiltration and sterile filtration.

The food enzyme is then formulated as a liquid or solid product.

The absence of the production microorganism in the food enzyme was demonstrated in [redacted]

No recombinant DNA was detected in three independent batches in triplicate [redacted]

The Panel considered the information provided on the raw materials and manufacturing process as sufficient.

3.1.6. Safety for the environment

The production strain and its recombinant DNA were not detected in the final product. Accordingly, no environmental risk assessment is required (EFSA GMO Panel, 2011).

3.1.7. Case of need and intended conditions of use

In the original submission, the intended uses of the food enzyme were: starch processing for glucose syrup production, baking, cereal based and brewing processes. In the course of the evaluation, the applicant informed EFSA about withdrawal of use of the food enzyme in cereal based and brewing processes.

The intended uses and the recommended use levels are summarised in Table 2.

[4] Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of food additives. OJ L 226, 25.6.2004, p. 3–21.

Table 2: Intended uses and recommended use levels of the food enzyme as provided by the applicant

Food manufacturing process[(a)]	Raw material	Recommended dosage of the food enzyme
Baking processes[(b)]	Flour	Up to 5,000 MANU/kg flour corresponding to 15 mg TOS/kg flour
Starch processing for glucose syrups production	Starch	Up to 16,500 MANU/kg starch corresponding to 49.5 mg TOS/kg starch

MANU: Maltogenic Amylase Novo Units; TOS: Total Organic Solids.
(a): The description provided by the applicant has been harmonised by EFSA according to the 'EC working document describing the food processes in which food enzymes are intended to be used' – not yet published at the time of adoption of this opinion.
(b): Restricted to bread-making including buns, scones and brioches.

In baking processes, the food enzyme is added during the preparation of the dough. It is used to hydrolyse starch and related polysaccharides to delay the staling process.

In starch processing for glucose syrups production, the food enzyme is added during the saccharification step. It is used to degrade starch polysaccharides into maltose and glucose in an efficient way.

3.1.8. Reaction and fate in food

The maltogenic amylase catalyses the hydrolysis of (1→4)-α-D-glucosidic linkages in amylose, amylopectin and related glucose polymers, liberating maltose units from the non-reducing end of the polymer chain.

Experimental data on the removal (> 99%) of protein in the course of starch processing for glucose syrups production have been provided (Documentation provided to EFSA No 3). The Panel considered the evidence as sufficient to conclude that residual amounts of TOS are removed by the purification steps applied during the production of glucose syrups, i.e. filtration, ion exchange chromatography, carbon treatment, crystallisation.

The data and information provided indicate that the maltogenic amylase is inactivated during the baking processes under the intended conditions of use.

3.2. Dietary exposure

Exposure estimates were calculated using the methodology described in the CEF Panel statement on the exposure assessment of food enzymes (EFSA CEF Panel, 2016). The assessment of the food process covered in this opinion involved selection of relevant food groups and application of process and technical conversion factors (Appendix B). These input data were subject to a stakeholder consultation through open calls,[5] and adjusted in accordance with feedback received.

3.2.1. EFSA Comprehensive European Food Consumption Database

Since 2010, the EFSA Comprehensive European Food Consumption Database (hereafter the EFSA Comprehensive Database[6]) has been populated with detailed national data on food consumption. Competent authorities in European countries provide EFSA with data regarding the level of food consumption by individual consumers, as taken from the most recent national dietary survey in their country (EFSA, 2011a).

The food consumption data gathered by EFSA were collected using different methodologies and thus direct country-to-country comparisons should be made with caution. Depending on the food category and the level of detail used in exposure calculations, uncertainties might be introduced owing to subjects possibly underreporting and/or misreporting of consumption amounts. Nevertheless, the EFSA Comprehensive Database is the best available source of food consumption data across Europe.

Food consumption data from the population groups: infants, toddlers, children, adolescents, adults and the elderly were used for the exposure assessment. For the present assessment, food

[5] http://www.efsa.europa.eu/en/data/call/161110
[6] http://www.efsa.europa.eu/en/food-consumption/comprehensive-database

consumption data were available from 33 different dietary surveys carried out in 19 European countries (Appendix A).

Consumption records were codified according to the FoodEx classification system (EFSA, 2011b).

3.2.2. Exposure assessment methodology

Chronic exposure was calculated based on individual consumption, averaged over the total survey period, excluding surveys with only one day per subject. High-level exposure/intake was calculated for only those population groups, in which the sample size was sufficiently large to allow calculation of the 95th percentile (EFSA, 2011a).

The exposure per FoodEx category was subsequently added to derive an individual total exposure per day. Finally, these exposure estimates were averaged over the number of survey days and normalised for individual body weight (bw), resulting in an individual average exposure/day per kg bw for the survey period. This was done for all individuals in the survey and per age class, resulting in distributions of individual average exposure per survey and age class. Based on these distributions, the mean and 95th percentile exposures were calculated per survey for the total population and per age class.

3.2.3. Exposure to food enzyme–TOS according to the intended use proposed by the applicant

Exposure to the food enzyme–TOS was based on intended uses and the recommended maximum use levels of the food enzyme–TOS provided by the applicant (Table 2). Foods/ingredients derived through starch processing, i.e. glucose syrups, were excluded from the analysis, as the Panel considered the presence of residual amounts of TOS in glucose syrups as negligible (see Section 3.1.7). Therefore, food enzyme–TOS exposure was calculated from foods produced involving a baking process only. The applicant proposed a restricted number of baking processes (Table 2), however, the Panel decided to follow the exposure methodology described in Section 3.2, i.e. inclusion of all baking applications, since no need for refining the exposure was identified.

Relevant food groups and/or individual foods were selected from the Comprehensive Database and were assumed to always contain the food enzyme–TOS at the maximum recommended use level. This will result in an overestimation of exposure to food enzyme–TOS.

To facilitate matching of the reported use levels for baking process with foods identified in the Comprehensive Database, the selected foods were disaggregated to ingredient level as appropriate, and converted into the corresponding raw material, i.e. flour, via the application of conversion factors (Appendix B). For example, consumption of 100 g of bread was converted into an intake of 70 g flour (recipe fraction of 0.7) and then multiplied by 15.0 mg TOS/kg flour, as provided by the applicant, to arrive at an exposure of 1.05 mg TOS.

Exposure to the food enzyme–TOS was calculated by multiplying values reported for each food category by their respective consumption amount per kilogram of body weight (kg bw) separately for each individual in the database. Table 3 provides an overview of the derived exposure estimates. The average and 95th percentile exposure to the food enzyme–TOS per age class, country and survey are reported in Appendix C – Table 1. The contribution of the food enzyme–TOS from each FoodEx category to the total dietary exposure is indicated in Appendix C – Table 2.

Table 3: Summary of estimated dietary exposure to food enzyme–TOS in six population groups

Population group	Estimated exposure (mg/kg bw per day)					
	Infants	**Toddlers**	**Children**	**Adolescents**	**Adults**	**The elderly**
Age range	3–11 months	12–35 months	3–9 years	10–17 years	18–64 years	≥ 65 years
Min–max mean (number of surveys)	0.010–0.046 (6)	0.039–0.095 (10)	0.041–0.090 (18)	0.025–0.060 (17)	0.018–0.036 (17)	0.017–0.031 (14)
Min–max 95th percentile (number of surveys)	0.061–0.129 (5)	0.089–0.160 (7)	0.077–0.168 (18)	0.044–0.118 (17)	0.035–0.071 (17)	0.032–0.057 (14)

bw: body weight.

3.2.4. Uncertainty analysis

In accordance with the guidance provided in the EFSA Opinion related to uncertainties in dietary exposure assessment (EFSA, 2006), the following sources of uncertainties have been considered and are summarised in Table 4.

Table 4: Qualitative evaluation of the influence of uncertainties on the dietary exposure estimate

Sources of uncertainties	Direction of impact
	Exposure to food enzyme–TOS
Model input data	
Consumption data: different methodologies/representativeness/underreporting/misreporting/no portion size standard	+/–
Use of data from food consumption survey of a few days to estimate long-term (chronic) exposure for high percentiles (95th percentile)	+
Possible national differences in categorisation and classification of food	+/–
Model assumptions and factors	
The estimation considered all food groups involving baking processes (e.g. breads and cakes)	+
FoodEx categories included in the exposure assessment were assumed to always contain the food enzyme–TOS	+
Exposure to food enzyme–TOS was always calculated based on the recommended maximum use level	+
Selection of broad FoodEx categories for the exposure assessment	+
Use of recipe fractions in disaggregation FoodEx categories likely to contain the food enzyme	+/–
Use of technical factors in the exposure model	+/–

+: uncertainty with potential to cause overestimation of exposure; –: uncertainty with potential to cause underestimation of exposure.

The conservative approach applied to the exposure estimate to food enzyme–TOS, in particular, assumptions made on the occurrence and use levels of this specific food enzyme, is likely to have led to a considerable overestimation of the exposure.

3.3. Toxicological data

The batch used for toxicological testing is an enzyme concentrate without addition of additives or other standardisation or stabilisation ingredients. It has been produced in accordance with the methods used for commercial batches, but the concentration has been done by means of evaporation instead of by ultrafiltration. The evaporation step removes water and thereby relatively increases non-enzymatic organic matter compared to activity. Table 1 shows that the food enzyme batch 4 used for the toxicological assays has the lowest specific activity (enzyme activity/mg TOS), which indicates that it is less pure than the commercial batches and thus can be considered as a 'worst-case' situation. Consequently, on the basis of the data provided, batch 4 is considered cruder than the three batches for commercialisation and its use for toxicological testing is considered acceptable.

3.3.1. Genotoxicity

3.3.1.1. Bacterial reverse mutation test

To investigate the potential of the maltogenic amylase to induce gene mutations in bacteria, a bacterial reverse mutation assay (Ames test) was performed according to OECD Test Guideline 471 (OECD, 1997a) and following Good Laboratory Practice (GLP). Four strains of *Salmonella* Typhimurium (TA1535, TA100, TA1537 and TA98) and *Escherichia coli* WP2uvrA were used in the presence or absence of metabolic activation with S9-mix, applying the direct plate incorporation method. Two separate experiments were carried out using six different concentrations (0, 156, 313, 625, 1,250, 2,500 and 5,000 μg dry matter/plate) of the food enzyme, appropriate positive control chemicals and deionised water as a negative control. The concentrations tested corresponded to ca. 0, 102, 205,

409, 818, 1,637 and 3,273 μg TOS/plate. All positive controls induced significant increases in revertant colony numbers, confirming the sensitivity of the tests and the efficacy of the S9-mix. Upon treatment with the food enzyme, bacteriotoxic effects were not observed in this study. Small non-reproducible increases in the number of colonies without dose relation were observed in few test series with S9 mix. These increases were not considered toxicologically relevant. Upon treatment with the food enzyme, there was no evidence of mutagenic activity of the food enzyme in this mutation test.

The Panel concluded that the food enzyme maltogenic amylase did not induce gene mutations in the bacterial reverse mutation assay under the test conditions employed in this study.

3.3.1.2. *In vitro* mammalian chromosomal aberration test

The *in vitro* chromosome aberration test was carried out according to the OECD Test Guideline 473 (OECD, 1997b) and following GLP. Cultures of peripheral blood human lymphocytes were prepared from the pooled blood of three female donors. The lymphocytes, proliferation of which was stimulated with phytohaemagglutinin (PHA), were treated with the food enzyme, sterile water (negative control) or appropriate positive controls in the absence or the presence of the S9-mix. Two experiments were performed. In the first experiment, applying 3 + 17 h treatment, the cultures were exposed to the food enzyme at concentrations of 3,200, 4,000 and 5,000 μg food enzyme/mL (corresponding to ca 291, 364 and 455 μg TOS/mL) in the presence and absence of the S9-mix. In the second experiment, applying continuous 20 + 0 h treatment without metabolic activation, concentrations scored for the chromosome aberrations were 1,886, 2,219 and 3,071 μg food enzyme/mL (corresponding to ca 172, 202 and 279 μg TOS/mL). For the short-term treatment (3 + 17 h) with metabolic activation, concentrations of 3,613, 4,250 and 5,000 μg food enzyme/mL (corresponding to 329, 387 and 455 μg TOS/mL) were tested. Reductions in the mitotic index of 47% and 1% were observed at 3,071 and 5,000 μg food enzyme/mL in the long-term treatment without S9-mix and the second short-term treatment in the presence of the S9-mix, respectively. Only cells with 44–46 chromosomes were analysed for chromosome aberrations, polyploidy and endoreduplication. Two hundred metaphases were analysed at each concentration. For all food enzyme concentrations used, the frequency of cells with chromosomal aberrations was similar to that of negative controls (values of $p \leq 0.05$ were considered as significant), except for 4,250 μg of food enzyme/mL in the second experiment in pulse 3 + 17 h treatment in the presence of S9-mix. As this effect was not reproducible, not concentration related, and found in one culture only, it is not considered to be of biological relevance.

The Panel concluded that the food enzyme maltogenic amylase did not induce chromosome aberrations, polyploidy and or endoreduplication in cultured human peripheral blood lymphocytes when tested under the test conditions employed in this study.

3.3.2. Repeated dose 90-day oral toxicity study in rodents

A repeated dose 90-day oral toxicity study was performed in accordance with OECD Test Guideline 408 (OECD, 1998) and following GLP. Groups of 10 male and 10 female Sprague–Dawley rats received the food enzyme orally via gavage volume of 10 mL/kg bw per day, corresponding to 97, 320 or 968 mg TOS/kg bw per day (referred to as low, mid and high dose groups). The control group received the vehicle water.

Food consumption of treated females was sporadically higher than that of the control animals and total food consumption of the low- and mid-dose females was significantly higher than that of the controls. This was reflected in a tendency for mean body weights of all treated females to be higher than that of the control animals from approximately day 15 but these differences seemed to be due partly to the marked contribution of individual animals to the mean group values. There was no dosage-related response, and group mean body weight gain of the food enzyme groups was within 15% of the control value. Although the food consumption of all treated males tended to be below that of the control group and significantly in the last 2 weeks of dosage, no significant difference was seen either between the tested groups or in the weight gain of the animals. The differences in food consumption were considered not to be toxicologically relevant.

At termination of the study, the relative neutrophil count (% of white blood cells) of high-dose males and females and the absolute neutrophil count of high-dose males and mid-dose females were significantly higher than those of the control groups. Relative, but not absolute lymphocytes were decreased and relative, but not absolute monocytes were significantly increased in high-dose males. No dose dependency was observed and values were within historical controls. Fibrinogen was

marginally increased in all treated females but there was no effect on clotting time. The haematological changes were not considered of toxicological importance.

A few statistically significant differences in clinical chemistry parameters were seen between the control group and the treated groups. The effects included increased urea and phosphorus in high-dose males, increased AST in high-dose females and increased beta protein in all treated females, but without a clear dose–response relationship.

Significant differences were seen between the control groups and the treated groups in urinalysis. The effects were increased epithelial cells in low- and high-dose males, increased *N*-acetyl-β-D-glucosaminidase (NAG)/mmol creatinine in high-dose females, increased leucocytes and epithelial cells in mid-dose females. The relative kidney weight of high-dose males and the absolute kidney weight of high-dose females were significantly higher than those of the control groups. Although no histopathological findings were observed at necropsy, the Panel decided as a conservative approach to select the mid-dose of 320 mg TOS/kg bw per day as the NOAEL of this study, since at the higher dose, statistically significant changes were observed in several kidney relevant parameter, among which NAG, which is an indicator or kidney damage.

A comparison of the NOAEL (320 mg TOS/kg bw per day) from the 90-day study with the derived exposure estimates in six human population groups of 0.010–0.095 mg TOS/kg bw per day at the mean and from 0.032 to 0.168 mg TOS/kg bw per day at the 95th percentile, resulted in margins of exposures (MOEs) above 1,905, indicating that there is no toxicological concern.

3.4. Allergenicity

The allergenicity assessment considers only the food enzyme and not any carrier or other excipient which may be used in the final formulation.

The allergenicity of maltogenic amylase produced with the genetically modified *Bacillus subtilis* strain NZYM-SM was assessed by comparing its amino acid sequence with those of known allergens according to the EFSA Scientific opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed of the Scientific Panel on Genetically Modified Organisms (EFSA GMO Panel, 2010). Using higher than 35% identity in a sliding window of 80 amino acids as criterion, three matches were found. The matching allergens are: Asp o 21, an α-amylase produced by *Aspergillus oryzae*; Asp f 13, a serine protease produced by *Aspergillus fumigatus*; and Sch c 1, a glucoamylase produced by *Schizophyllum commune*.

α-Amylase from *A. oryzae* (Brisman and Belin, 1991; Brisman, 2002), serine protease from *A. fumigatus* (Kurup et al., 2002) and glucoamylase from *Schizophyllum commune* (Sander et al., 1998; Quirce et al., 2002) are all described as occupational respiratory allergens associated with baker's asthma. However, several studies have shown that adults with occupational asthma to a food enzyme (like α-amylase) can commonly ingest the corresponding enzyme without acquiring clinical symptoms of food allergy (Cullinan et al., 1997; Brisman, 2002; Poulsen, 2004; Armentia et al., 2009). Taking into account the wide use of α-amylase, only a low number of case reports have been described in literature focussed on allergic reactions upon oral exposure to α-amylase in individuals respiratory sensitised to α-amylase (Baur and Czuppon, 1995; Kanny and Moneret-Vautrin, 1995; Moreno-Ancillo et al., 2004). In addition, such information is not reported for serine protease and glucoamylase. Therefore, it can be concluded that an allergic reaction upon oral ingestion of maltogenic amylase, produced by the genetically modified *B. subtilis* strain NZYM-SM, in individuals respiratory sensitised to α-amylase, serine protease produced by *A. fumigatus* or glucoamylase produced by *Schizophyllum commune* cannot be excluded, but the likelihood is considered to be low. Moreover, no information is available on oral sensitisation or elicitation reactions of this maltogenic amylase.

Bindslev-Jensen et al. (2006) investigated the cross reactivity of 19 different commercial enzymes used in the food industry in allergic patients (400 patients allergic to inhalation allergens, food allergens, allergens of bee or wasp or drugs). A maltogenic amylase from a *B. subtilis* species only gave a positive skin prick test in two allergic patients. Nevertheless, it was further tested by ingestion (DBPCFC) and was found to be negative to both active and placebo.

According to the information provided, substances or products that may cause allergies ([redacted]) or intolerances (Regulation EU 1169/2011)[7] are used as raw materials in the media fed to the microorganisms. However, these substances will be degraded and utilised by the microorganisms for cell growth, cell maintenance and production of enzyme. In addition, the microbial biomass and fermentation solids will be removed. Therefore, potentially allergenic residues of these foods employed as protein sources are not expected to be present.

Taken together, the Panel considers that there are no indications for allergic reactions by dietary exposure to the food enzyme maltogenic amylase produced with the genetically modified *B. subtilis* strain NZYM-SM.

Conclusions

Based on the genetic modifications performed, the manufacturing process, the compositional and biochemical data provided, the dietary exposure assessment and the findings in the toxicological studies and the allergenicity assessment, the Panel concluded that the food enzyme maltogenic amylase from *Bacillus subtilis* strain NZYM-SM does not give rise to safety concerns under the intended conditions of use.

Recommendations

Documentation provided to EFSA

1) Dossier 'Maltogenic amylase produced by a genetically modified strain of *Bacillus subtilis* (strain NZYM-SM)'. February 2015. Submitted by Novozymes A/S.
2) Summary report on genotoxicity and subchronic toxicity study related to maltogenic alpha-amylase produced with a strain of *Bacillus subtilis* (strain NZYM-SM). March 2016. Delivered by FoBiG GmbH, Freiburg (Germany).
3) Additional information on 'Food enzyme carry/over in glucose syrups'. February 2017. Provided by the Association of Manufacturers and Formulators of Enzyme Products.
4) Additional information was received from Novozymes A/S in November 2017.
5) Additional information was received from Novozymes A/S in January 2018.

References

Armentia A, Dias-Perales A, Castrodeza J, Dueñas-Laita A, Palacin A and Fernandes S, 2009. Why can patients with baker's asthma tolerate wheat flour ingestion? Is wheat pollen allergy relevant? Allergologia et Immunopathologia, 37, 203–204.

Baur X and Czuppon AB, 1995. Allergic reaction after eating alpha-amylase (Asp o 2)-containing bread. A case report. Allergy, 50, 85–87.

Bindslev-Jensen C, Skov PS, Roggen EL, Hvass P and Brinch DS, 2006. Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry. Food and Chemical Toxicology, 44, 1909–1915.

Brisman J, 2002. Baker's asthma. Occupational and Environmental Medicine, 59, 498–502; quiz 502, 426.

Brisman J and Belin L, 1991. Clinical and immunological responses to occupational exposure to alpha-amylase in the baking industry. British Journal of Industrial Medicine, 48, 604–608.

Cullinan P, Cook A, Jones M, Cannon J, Fitzgerald B and Newman Taylor AJ, 1997. Clinical responses to ingested fungal a-amylase and hemicellulase in persons sensitized to *Aspergillus fumigatus*? Allergy, 52, 346–349.

EFSA (European Food Safety Authority), 2006. Opinion of the Scientific Committee related to uncertainties in dietary exposure assessment. EFSA Journal 2006;5(1):438, 54 pp. https://doi.org/10.2903/j.efsa.2007.438

EFSA (European Food Safety Authority), 2009a. Guidance of EFSA prepared by the Scientific Panel of Food Contact Material, Enzymes, Flavourings and Processing Aids on the Submission of a Dossier on Food Enzymes. EFSA Journal 2009;7(5):1305, 26 pp. https://doi.org/10.2903/j.efsa.2009.1305

EFSA (European Food Safety Authority), 2009b. Guidance of the Scientific Committee on transparency in the scientific aspects of risk assessments carried out by EFSA. Part 2: general principles. EFSA Journal 2009;7(5):1051, 22 pp. https://doi.org/10.2903/j.efsa.2009.1051

EFSA (European Food Safety Authority), 2011a. Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment. EFSA Journal 2011;9(3):2097, 34 pp. https://doi.org/10.2903/j.efsa.2011.2097

[7] REGULATION (EU) No 1169/2011 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004.

EFSA (European Food Safety Authority), 2011b. Evaluation of the FoodEx, the food classification system applied to the development of the Comprehensive European Food Consumption Database. EFSA Journal 2011;9(3):1970, 27 pp. https://doi.org/10.2903/j.efsa.2011.1970

EFSA CEF Panel (EFSA Panel on Food Contact Material, Enzymes, Flavourings and Processing Aids), 2009. Guidance of the Scientific Panel of Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) on the Submission of a Dossier on Food Enzymes for Safety Evaluation by the Scientific Panel of Food Contact Material, Enzymes, Flavourings and Processing Aids. EFSA Journal 2009;1305, 1–26. https://doi.org/10.2903/j.efsa.2009.1305

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), Silano V, Bolognesi C, Castle L, Cravedi J-P, Fowler P, Franz R, Grob K, G€urtler R, Husøy T, Karenlampi S, Mennes W, Milana MR, Penninks A, Smith A, Tavares Pocas MF, Tlustos C, Wolfle D, Zorn H, Zugravu C-A, Arcella D, Liu Y and Engel K-H, 2016. Panel statement on the exposure assessment of food enzymes. EFSA Journal 2016;14(11):4581, 9 pp. https://doi.org/10.2903/j.efsa.2016.4581

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2010. Scientific opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed. EFSA Journal 2010;8(7):1700. 168 pp. https://doi.org/10.2903/j.efsa.2010

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2011. Scientific Opinion on Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use. EFSA Journal 2011;9(6):2193, 54 pp. https://doi.org/10.2903/j.efsa.2011.2193

FAO/WHO (Food and Agriculture Organization of the United Nations/World Health Organization), 2006. General specifications and considerations for enzyme preparations used in food processing in Compendium of food additive specifications. 67th meeting. Joint FAO/WHO Expert Committee on Food Additives Monographs 3, 63–67. Available online: ftp://ftp.fao.org/docrep/fao/009/a0675e/a0675e00.pdf

[redacted]

Kanny G and Moneret-Vautrin D-A, 1995. Alpha-amylase contained in bred can induce food allergy. Journal of Allergy Clinical and Immunology, 95, 132–133.

Kurup VP, Xia JQ, Shen HD, Rickaby DA, Henderson Jr JD, Fink JN, Chou H, Kelly KJ and Dawson CA. 2002. Alkaline serine proteinase from *Aspergillus fumigatus* has synergistic effects on Asp-f-2-induced immune response in mice. International Archives of Allergy and Immunology, 129, 129–137.

Moreno-Ancillo A, Domınguez-Noche C, Gil-Adrados AC and Cosmes PM, 2004. Bread eating induced oral angioedema due to alpha-amylase allergy. Journal of Investigative Allergology and Clinical Immunology, 14, 346–347.

OECD (Organisation for Economic Co-operation and Development), 1997a. Bacterial Reverse Mutation Test. Guideline 471. Adopted 21.7.1997. Available online: http://www.oecd-ilibrary.org/environment/test-no-471-bacterial-reverse-mutation-test_9789264071247-en;jsessionid=9zfgzu35paaq.x-oecd-live-01

OECD (Organisation for Economic Co-operation and Development), 1997b. In vitro Mammalian Chromosomal Aberration Test. Guideline 473. Adopted 21.7.1997. Available online: http://www.oecd-ilibrary.org/environment/test-no-473-in-vitromammalian-chromosome-aberration-test_9789264071261-en

OECD (Organisation for Economic Co-operation and Development), 1998. Repeated Dose 90-Day Oral Toxicity Study in Rodents. Guideline 408. Adopted 21.09.1998. ISBN: 9789264070707 (PDF) https://doi.org/10.1787/9789264070707-en. Available online: http://www.oecd-ilibrary.org/environment/test-no-408-repeated-dose-90-day-oral-toxicity-study-in-rodents_9789264070707-en

Pedersen PB, Bjørnvad ME, Rasmussen MD and Petersen JN, 2002. Cytotoxic potential of industrial strains of *Bacillus* sp. Regulatory Toxicology and Pharmacology, 36, 155–161.

Poulsen LK, 2004. Allergy assessment of foods or ingredients derived from biotechnology, gene-modified organisms, or novel food. Molecular Nutrition and Food Research, 48, 413–423.

Quirce S, Fernandez-Nieto M, Bartolome B, Bombin C, Cuevas M, Sastre J, 2002. Glucoamylase: another fungal enzyme associated with baker's asthma. Annals of Allergy, Asthma and Immunology, 89, 197–202.

Sander I, Raulf-Heimsoth M, Siethoff C, Lohaus C, Meyer HE, Baur X, 1998. Allergy to *Aspergillus*-derived enzymes in the baking industry: identification of beta-xylosidase from *Aspergillus niger* as a new allergen (Asp n 14). Journal of Allergy Clinical and Immunology, 102, 256–264.

Abbreviations

bw	body weight
CAS	Chemical Abstracts Service
CFU	colony forming units
DBPCFC	double blind, placebo controlled food challenge
[redacted]	[redacted]

EC	Enzyme Commission
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organization
GLP	Good Laboratory Practice
GMO	genetically modified organism
GMP	Good Manufacturing Practice
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LOD	limit of detection
LOQ	limit of quantification
MOE	margin of exposure
NAG	*N*-acetyl-β-D-glucosaminidase
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Cooperation and Development
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
SDS–PAGE	sodium dodecyl sulfate–poly acrylamide gel electrophoresis
TOS	Total Organic Solids
WHO	World Health Organization

Appendix A – Population groups considered for the exposure assessment

Population	Age range	Countries with food consumption surveys covering more than one day
Infants	From 12 weeks on up to and including 11 months of age	Bulgaria, Denmark, Finland, Germany, Italy, United Kingdom
Toddlers	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Denmark, Finland, Germany, Italy, Netherlands, Spain, United Kingdom
Children[(a)]	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Czech Republic, Denmark, Finland, France, Germany, Greece, Italy, Latvia, Netherlands, Spain, Sweden, United Kingdom
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Cyprus, Czech Republic, Denmark, Finland, France, Germany, Italy, Latvia, Spain, Sweden, United Kingdom
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, Netherlands, Romania, Spain, Sweden, United Kingdom
The elderly[(a)]	From 65 years of age and older	Austria, Belgium, Denmark, Finland, France, Germany, Hungary, Ireland, Italy, Romania, Sweden, United Kingdom

(a): The terms 'children' and 'the elderly' correspond, respectively, to 'other children' and the merge of 'elderly' and 'very elderly' in the Guidance of EFSA on the 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011a).

Appendix B – FoodEx categories used to derive exposure estimates for the food enzyme–TOS and the respective conversion factors

FoodEx code	FoodEx category	Conversion factor from FoodEx food group to raw material[a]	Recipe fraction[b]	mg TOS/ kg flour
A.01	Grains and grain-based products (unspecified)	0.8	1	15.0
A.01.03	Grain milling products (unspecified)	1	1	15.0
A.01.03.001	Wheat milling products (unspecified)	1	1	15.0
A.01.03.001.001	Wheat flour, brown	1	1	15.0
A.01.03.001.002	Wheat flour, Durum	1	1	15.0
A.01.03.001.003	Wheat flour, white	1	1	15.0
A.01.03.001.004	Wheat flour, wholemeal	1	1	15.0
A.01.03.001.005	Graham flour	1	1	15.0
A.01.03.001.006	Wheat flour, gluten free	1	1	15.0
A.01.03.001.014	Wheat starch	1.2	1	15.0
A.01.03.002	Rye milling products (unspecified)	1	1	15.0
A.01.03.002.001	Rye flour, gluten free	1	1	15.0
A.01.03.002.002	Rye flour, light	1	1	15.0
A.01.03.002.003	Rye flour, medium	1	1	15.0
A.01.03.002.004	Rye flour, wholemeal	1	1	15.0
A.01.03.003	Buckwheat milling products (unspecified)	1	1	15.0
A.01.03.003.001	Buckwheat flour	1	1	15.0
A.01.03.004	Corn milling products (unspecified)	1	1	15.0
A.01.03.004.001	Corn flour	1	1	15.0
A.01.03.004.003	Corn starch	1.3	1	15.0
A.01.03.005	Oat milling products (unspecified)	1	1	15.0
A.01.03.005.002	Oat flour	1	1	15.0
A.01.03.005.004	Oat starch	1.2	1	15.0
A.01.03.006	Rice milling products (unspecified)	1	1	15.0
A.01.03.006.001	Rice flour	1	1	15.0
A.01.03.006.002	Rice flour white	1	1	15.0
A.01.03.006.003	Rice flour, instant	1	1	15.0
A.01.03.006.004	Rice starch	1.2	1	15.0
A.01.03.007	Spelt milling products	1	1	15.0
A.01.03.008	Other milling products (unspecified)	1	1	15.0
A.01.03.008.001	Amaranth flour	1	1	15.0
A.01.03.008.002	Barley flour	1	1	15.0
A.01.03.008.003	Chapatti flour	1	1	15.0
A.01.03.008.004	Flour mix, wheat/rye/barley/oats	1	1	15.0
A.01.03.008.005	Millet flour	1	1	15.0
A.01.03.008.007	Sorghum flour	1	1	15.0
A.01.04	Bread and rolls (unspecified)	1	0.7	15.0
A.01.04.001	Wheat bread and rolls	1	0.7	15.0
A.01.04.002	Rye bread and rolls	1	0.7	15.0
A.01.04.003	Mixed wheat and rye bread and rolls	1	0.7	15.0
A.01.04.004	Multigrain bread and rolls	1	0.7	15.0
A.01.04.005	Unleavened bread, crisp bread and rusk (unspecified)	1	0.8	15.0
A.01.04.005.001	Crisp bread, rye wholemeal	1	0.9	15.0

FoodEx code	FoodEx category	Conversion factor from FoodEx food group to raw material[a]	Recipe fraction[b]	mg TOS/ kg flour
A.01.04.005.002	Crisp bread, rye, light	1	0.9	15.0
A.01.04.005.003	Crisp bread, wheat, wholemeal	1	0.9	15.0
A.01.04.005.004	Crisp bread, wheat, light	1	0.9	15.0
A.01.04.005.005	Rusk, light	1	0.9	15.0
A.01.04.005.006	Rusk, wholemeal	1	0.9	15.0
A.01.04.005.007	Pita bread	1	0.7	15.0
A.01.04.005.008	Matzo	1	0.9	15.0
A.01.04.005.009	Tortilla	1	0.7	15.0
A.01.04.006	Other bread	1	0.7	15.0
A.01.04.007	Bread products	1	0.7	15.0
A.01.07	Fine bakery wares (unspecified)	1	0.5	15.0
A.01.07.001	Pastries and cakes (unspecified)	1	0.5	15.0
A.01.07.001.001	Beignets	1	0.15	15.0
A.01.07.001.002	Buns	1	0.7	15.0
A.01.07.001.003	Cake from batter	1	0.25	15.0
A.01.07.001.004	Cheese cream cake	1	0.24	15.0
A.01.07.001.005	Cheese cream sponge cake	1	0.24	15.0
A.01.07.001.006	Chocolate cake	1	0.24	15.0
A.01.07.001.007	Chocolate cake with fruits	1	0.24	15.0
A.01.07.001.008	Cream cake	1	0.24	15.0
A.01.07.001.009	Cream cheese cake	1	0.24	15.0
A.01.07.001.010	Cream custard cake	1	0.24	15.0
A.01.07.001.011	Cream custard sponge cake	1	0.24	15.0
A.01.07.001.012	Croissant	1	0.5	15.0
A.01.07.001.013	Croissant, filled with chocolate	1	0.5	15.0
A.01.07.001.014	Croissant, filled with cream	1	0.5	15.0
A.01.07.001.015	Croissant, filled with jam	1	0.5	15.0
A.01.07.001.016	Croquembouche	1	0.15	15.0
A.01.07.001.017	Doughnuts	1	0.24	15.0
A.01.07.001.018	Clair	1	0.15	15.0
A.01.07.001.019	Flan	1	0.5	15.0
A.01.07.001.020	Fruit cake	1	0.6	15.0
A.01.07.001.021	Fruit pie	1	0.15	15.0
A.01.07.001.022	Cheese pie	1	0.15	15.0
A.01.07.001.023	Fruit tart	1	0.15	15.0
A.01.07.001.024	Gingerbread	1	0.6	15.0
A.01.07.001.025	Gougere	1	0.15	15.0
A.01.07.001.026	Kringles	1	0.25	15.0
A.01.07.001.027	Nut cream cake	1	0.24	15.0
A.01.07.001.028	Pancakes	1	0.25	15.0
A.01.07.001.029	Profiterole	1	0.15	15.0
A.01.07.001.030	Pyramid cake	1	0.25	15.0
A.01.07.001.031	Rhubarb flan	1	0.15	15.0
A.01.07.001.032	Scone	1	0.5	15.0
A.01.07.001.033	Sponge dough	1	0.25	15.0
A.01.07.001.034	Sponge cake	1	0.25	15.0
A.01.07.001.035	Sponge cake roll	1	0.25	15.0

FoodEx code	FoodEx category	Conversion factor from FoodEx food group to raw material[a]	Recipe fraction[b]	mg TOS/ kg flour
A.01.07.001.036	Muffins	1	0.25	15.0
A.01.07.001.037	Waffles	1	0.25	15.0
A.01.07.001.038	Apple strudel	1	0.15	15.0
A.01.07.001.039	Cream-cheese strudel	1	0.24	15.0
A.01.07.001.040	Cheese pastry goods from puff pastry	1	0.15	15.0
A.01.07.001.041	Croissant from puff pastry	1	0.6	15.0
A.01.07.001.042	Brioche	1	0.5	15.0
A.01.07.001.044	Lebkuchen	1	0.6	15.0
A.01.07.001.045	Dumpling	1	0.5	15.0
A.01.07.001.046	Cake marbled, with chocolate	1	0.5	15.0
A.01.07.001.047	Marzipan pie	1	0.25	15.0
A.01.07.001.048	Baklava	1	0.15	15.0
A.01.07.002	Biscuits (cookies)	1	0.9	15.0
A.01.07.002.001	Biscuits, sweet, plain	1	0.9	15.0
A.01.07.002.002	Biscuits, chocolate filling	1	0.81	15.0
A.01.07.002.003	Biscuits, cream filling	1	0.81	15.0
A.01.07.002.004	Biscuits, fruit filling	1	0.81	15.0
A.01.07.002.005	Biscuits, vanilla filling	1	0.81	15.0
A.01.07.002.006	Butter biscuits	1	0.81	15.0
A.01.07.002.007	Biscuit, iced	1	0.81	15.0
A.01.07.002.008	Speculaas	1	0.9	15.0
A.01.07.002.009	Biscuits, sweet, wheat wholemeal	1	0.9	15.0
A.01.07.002.010	Biscuits, oat meal	1	0.9	15.0
A.01.07.002.011	Biscuits, spelt meal	1	0.9	15.0
A.01.07.002.012	Biscuits, salty	1	0.9	15.0
A.01.07.002.013	Biscuits, salty, with cheese	1	0.81	15.0
A.01.07.002.014	Sticks, salty	1	0.81	15.0
A.17.03.003	Biscuits, rusks and cookies for children	1	0.9	15.0
A.18.04.001	Find bakery products for diabetics	1	0.5	15.0
A.19.01.001	Sandwich and sandwich-like meal	1	0.32	15.0
A.19.01.002	Pizza and pizza-like pies	1	0.3	15.0

TOS: Total Organic Solids.
(a): Available at see http://www.fao.org/fileadmin/templates/ess/documents/methodology/tcf.pdf
(b): Derived from publically available recipe information, and/or food label information (such as the Mintel's Global New Products Database http://www.mintel.com/global-new-products-database).

Appendix C – Dietary exposure estimates to the food enzyme–TOS in details

Information provided in this appendix is shown in an excel file (downloadable http://onlinelibrary.wiley.com/wol1/doi/10.2903/j.efsa.2018.5171/suppinfo).

The file contains two sheets, corresponding to two tables.

Table 1: Average and 95th percentile exposure to the food enzyme–TOS per age class, country and survey.

Table 2: The contribution of the food enzyme–TOS from each FoodEx category to the total dietary exposure.

TÜV NORD GROUP
TUV INDIA PRIVATE LIMITED
TUV India House,
Survey No: 42, 3/1 & 3/2,
Sus, Tal. Mulshil,
Dist. Pune - 411 021
CIN : U74140MH1989PTC052930
Tel. : 020 - 67900000 / 01
Toll free :1800-209-0902
Email : pune@tuv-nord.com
Website : www.tuv-nord.com/in

TEST REPORT

Report No : TUV(I)/14187/21-22/0022200969
Date : 17 Feb 2022

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

Reg No. : 14187/21-22

CA No. : 0022200969

Date of sample receipt : 31 Jan 2022

Date(s) of analysis : 05 Feb 2022 - 17 Feb 2022

Sample Drawn by : Customer

SlNo	Test Name	Results	Unit	LOQ	Test Method
	Sample Name : Maltogenic Amylase Batch No.: 012244 [redacted]			**CA No : 0022200969** [redacted]	
[redacted]					
	Heavy Metals				
5	Lead	< LOQ	mg/kg	0.1	TUV/03/SOP/004 Based on AOAC 2015.01, 21 st Edition
6	Mercury	< LOQ	mg/kg	0.025	TUV/03/SOP/004 Based on AOAC 2015.01 21 st Edition
7	Arsenic	0.14	mg/kg	0.1	TUV/03/SOP/004 Based on AOAC 2015.01, 21 st Edition
8	Cadmium	< LOQ	mg/kg	0.1	TUV/03/SOP/004 Based on AOAC 2015.01 21 st Edition
[redacted]					

TEST REPORT

Report No : TUV(I)/14187/21-22/0022200969
Date : 17 Feb 2022

LOQ-Limit of Quantification

Verified by

Shaine Premkumar
Manager – Microbiology

Authorized by

Atulkumar Rajage
Manager – Instrumentation Department

Note - This is Electronically Generated Report Copy

Terms and Conditions

1. Test Results are based on & related only to the particular sample(s) tested.
2. This Report cannot be re-produced, except when in full, without the written permission from TUV India Pvt. Ltd.
3. This Certificate reflects our findings at the time and place of testing.
4. Sample(s) will be retained by us for a period of one month for non-perishable items only. Perishable items will be destroyed after completion of tests.
5. This Report, in full or in part, shall not be used to make any misleading claims or for any legal purposes.
6. All terms and conditions of our quotation on the basis of which this testing service has been provided are deemed to be fully accepted by the customer and are deemed to be in full force and effect.
7. This Report is exclusively for the use of the customer whose name and address is indicated above. No third party can derive rights against the company on the basis of this report. No third party has any right to raise any claims on the company.
8. For Biological and mycotoxin Analysis: Our analytical findings reflect the quality of the sample at the time of testing. No responsibility can be accepted for the possible consequences of further development of micro-organisms or mycotoxin which may depend upon storage, handling & weathers conditions which may influence the results at a later date/time respectively. For Coliform Count <10, Detection of Salmonella Spp <1 can be considered as absent.
9. The laboratory tests are conducted by TUV India as per mutual agreement without any implied warranty of merchantability and fitness for any general or particular use. TUV India will not be liable for any claims for indirect, incidental or consequential damages including but not limited to loss of revenue, loss of profits and similar claims. TUV India will also not be liable under any strict liability, product liability or negligence. The total liability, scientifically and legally proven, if any, of TUV India, in any case, shall not exceed the invoiced amount for the services provided and paid for.
10. For Pesticide Residue analysis, reported value at LOQ level may vary within analytical variation of 50% considering Uncertainty of Measurement.
11. This Test Report is not covered under NABL scope of Accreditation.

-- End of Report --

Registered & Head Office : 801, Raheja Plaza-1, L.B.S. Marg, Ghatkopar (W), Mumbai - 400 086
Phone : (022) 6647 7000 • Fax : (022) 6647 7009 • e-mail : mumbai@tuv-nord.com • website : www.tuvindia.co.in
TUV NORD GROUP

TÜV NORD GROUP
TUV INDIA PRIVATE LIMITED
TUV India House,
Survey No: 42, 3/1 & 3/2,
Sus, Tal. Mulshil,
Dist. Pune - 411 021
CIN : U74140MH1989PTC052930
Tel. : 020 - 67900000 / 01
Toll free :1800-209-0902
Email : pune@tuv-nord.com
Website : www.tuv-nord.com/in

TEST REPORT

Report No : TUV(I)/14187/21-22/0022200970
Date : 17 Feb 2022

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

Reg No. : 14187/21-22

CA No. : 0022200970

Date of sample receipt : 31 Jan 2022

Date(s) of analysis : 05 Feb 2022 - 17 Feb 2022

Sample Drawn by : Customer

SlNo	Test Name	Results	Unit	LOQ	Test Method
	Sample Name : Maltogenic Amylase Batch No.: 012246			**CA No : 0022200970**	
	[redacted]				
	Heavy Metals				
5	Lead	< LOQ	mg/kg	0.1	TUV/03/SOP/004 Based on AOAC 2015.01, 21 st Edition
6	Mercury	< LOQ	mg/kg	0.025	TUV/03/SOP/004 Based on AOAC 2015.01 21 st Edition
7	Arsenic	0.19	mg/kg	0.1	TUV/03/SOP/004 Based on AOAC 2015.01, 21 st Edition
8	Cadmium	< LOQ	mg/kg	0.1	TUV/03/SOP/004 Based on AOAC 2015.01 21 st Edition
	[redacted]				

TEST REPORT

Report No : TUV(I)/14187/21-22/0022200970
Date : 17 Feb 2022

LOQ-Limit of Quantification

Verified by

Shaine Premkumar
Manager – Microbiology

Authorized by

Atulkumar Rajage
Manager – Instrumentation Department

Note - This is Electronically Generated Report Copy

Terms and Conditions

1. Test Results are based on & related only to the particular sample(s) tested.
2. This Report cannot be re-produced, except when in full, without the written permission from TUV India Pvt. Ltd.
3. This Certificate reflects our findings at the time and place of testing.
4. Sample(s) will be retained by us for a period of one month for non-perishable items only. Perishable items will be destroyed after completion of tests.
5. This Report, in full or in part, shall not be used to make any misleading claims or for any legal purposes.
6. All terms and conditions of our quotation on the basis of which this testing service has been provided are deemed to be fully accepted by the customer and are deemed to be in full force and effect.
7. This Report is exclusively for the use of the customer whose name and address is indicated above. No third party can derive rights against the company on the basis of this report. No third party has any right to raise any claims on the company.
8. For Biological and mycotoxin Analysis: Our analytical findings reflect the quality of the sample at the time of testing. No responsibility can be accepted for the possible consequences of further development of micro-organisms or mycotoxin which may depend upon storage, handling & weathers conditions which may influence the results at a later date/time respectively. For Coliform Count <10 can be considered as absent.
9. The laboratory tests are conducted by TUV India as per mutual agreement without any implied warranty of merchantability and fitness for any general or particular use. TUV India will not be liable for any claims for indirect, incidental or consequential damages including but not limited to loss of revenue, loss of profits and similar claims. TUV India will also not be liable under any strict liability, product liability or negligence. The total liability, scientifically and legally proven, if any, of TUV India, in any case, shall not exceed the invoiced amount for the services provided and paid for.
10. For Pesticide Residue analysis, reported value at LOQ level may vary within analytical variation of 50% considering Uncertainty of Measurement.
11. This Test Report is not covered under NABL scope of Accreditation.

-- End of Report --

Registered & Head Office : 801, Raheja Plaza-1, L.B.S. Marg, Ghatkopar (W), Mumbai - 400 086
Phone : (022) 6647 7000 • Fax : (022) 6647 7009 • e-mail : mumbai@tuv-nord.com • website : www.tuvindia.co.in
TUV NORD GROUP

TÜV NORD GROUP
TUV INDIA PRIVATE LIMITED
TUV India House,
Survey No: 42, 3/1 & 3/2,
Sus, Tal. Mulshil,
Dist. Pune - 411 021
CIN : U74140MH1989PTC052930
Tel. : 020 - 67900000 / 01
Toll free :1800-209-0902
Email : pune@tuv-nord.com
Website : www.tuv-nord.com/in

TEST REPORT

Report No : TUV(I)/14187/21-22/0022200971
Date : 17 Feb 2022

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

Reg No. : 14187/21-22

CA No. : 0022200971

Date of sample receipt : 31 Jan 2022

Date(s) of analysis : 05 Feb 2022 - 17 Feb 2022

Sample Drawn by : Customer

SlNo	Test Name	Results	Unit	LOQ	Test Method
	Sample Name : Maltogenic Amylase Batch No.: 012248			**CA No : 0022200971**	
	[redacted]				
	Heavy Metals				
5	Lead	< LOQ	mg/kg	0.1	TUV/03/SOP/004 Based on AOAC 2015.01, 21 st Edition
6	Mercury	< LOQ	mg/kg	0.025	TUV/03/SOP/004 Based on AOAC 2015.01 21 st Edition
7	Arsenic	0.15	mg/kg	0.1	TUV/03/SOP/004 Based on AOAC 2015.01, 21 st Edition
8	Cadmium	< LOQ	mg/kg	0.1	TUV/03/SOP/004 Based on AOAC 2015.01 21 st Edition
	[redacted]				

TEST REPORT

Report No : TUV(I)/14187/21-22/0022200971
Date : 17 Feb 2022

LOQ-Limit of Quantification

Verified by

Shaine Premkumar
Manager – Microbiology

Authorized by

Atulkumar Rajage
Manager – Instrumentation Department

Note - This is Electronically Generated Report Copy

Terms and Conditions

1. Test Results are based on & related only to the particular sample(s) tested.
2. This Report cannot be re-produced, except when in full, without the written permission from TUV India Pvt. Ltd.
3. This Certificate reflects our findings at the time and place of testing.
4. Sample(s) will be retained by us for a period of one month for non-perishable items only. Perishable items will be destroyed after completion of tests.
5. This Report, in full or in part, shall not be used to make any misleading claims or for any legal purposes.
6. All terms and conditions of our quotation on the basis of which this testing service has been provided are deemed to be fully accepted by the customer and are deemed to be in full force and effect.
7. This Report is exclusively for the use of the customer whose name and address is indicated above. No third party can derive rights against the company on the basis of this report. No third party has any right to raise any claims on the company.
8. For Biological and mycotoxin Analysis: Our analytical findings reflect the quality of the sample at the time of testing. No responsibility can be accepted for the possible consequences of further development of micro-organisms or mycotoxin which may depend upon storage, handling & weathers conditions which may influence the results at a later date/time respectively. For Coliform Count <10 can be considered as absent.
9. The laboratory tests are conducted by TUV India as per mutual agreement without any implied warranty of merchantability and fitness for any general or particular use. TUV India will not be liable for any claims for indirect, incidental or consequential damages including but not limited to loss of revenue, loss of profits and similar claims. TUV India will also not be liable under any strict liability, product liability or negligence. The total liability, scientifically and legally proven, if any, of TUV India, in any case, shall not exceed the invoiced amount for the services provided and paid for.
10. For Pesticide Residue analysis, reported value at LOQ level may vary within analytical variation of 50% considering Uncertainty of Measurement.
11. This Test Report is not covered under NABL scope of Accreditation.

-- End of Report --

Registered & Head Office : 801, Raheja Plaza-1, L.B.S. Marg, Ghatkopar (W), Mumbai - 400 086
Phone : (022) 6647 7000 • Fax : (022) 6647 7009 • e-mail : mumbai@tuv-nord.com • website : www.tuvindia.co.in
TUV NORD GROUP

Query Response: GRN 1097

Maltogenic alpha-amylase from *Geobacillus stearothermophilus* produced by *Escherichia coli* BLASC (SD-6849)

1. **Please clarify the difference between the calculated (75.2 kDa) and experimentally determined (62kDa) molecular weight of the maltogenic alpha-amylase.**

   Response:

   The calculated molecular mass of maltogenic alpha amylase is 75.2 kDa, based on the amino acid sequence. However, we obtained a molecular weight of 62 kDa, when analysed by SDS-PAGE.

   Due to this discrepancy, we further determined the molecular mass by MALDI-TOF MS, and obtained a molecular mass of 75806.8 Daltons (75.8 kDa), which is in agreement with the theoretically calculated value.

   As per the published literature, maltogenic alpha amylase is known to give a much lower than expected molecular weight, when analysed by SDS-PAGE, for reasons not well known. Refer to the Annexure 1-EFSA (European Food Safety Authority) opinion on maltogenic amylase from GMM *B. subtilis* NZYM-SM [1] (EFSA Journal 2018;16(5):5171 [page 5, section 3.1.2]), where a similar maltogenic alpha amylase (from *Bacillus stearothermophilus*) was analysed by SDS-PAGE, and was found to migrate below 66 kDa, whereas the calculated molecular weight, based on the amino acid sequence is 75.2 kDa. However, exact molecular weight is not reported in this publication. Our SDS-PAGE data also indicates that the protein migrates just below the protein standard of 66 kDa.

2. **On page 22, you state "The raw materials used in fermentation conform to current Food Chemicals Codex specifications except for those that do not appear in the FCC" but do not provide a citation or specific FCC edition. For the administrative record, please provide a citation for the FCC edition referenced in the notice and confirm that the specifications conform to the most recent FCC edition (e.g. FCC 13).**

   Response:

   The raw materials used in fermentation for the production of maltogenic alpha amylase conform to current Food Chemicals Codex specifications (FCC13).

3. **Advanced Enzymes states that AOAC methods 984.27 and 999.10 were used for the determination of lead content. AOAC method 984.27 is validated for the determination of calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc in infant formula using ICP-OES. The method is not validated for the quantification of lead and thus it is not appropriate. AOAC method 999.10 is validated for lead, cadmium, zinc, copper and iron in food using AAS. Annex B of the notice states that the method used for the quantification of lead is ICP-MS. Please clarify the discrepancy and provide results from appropriate method such as FDA EAM 4.7 or AOAC 2015.01. In accordance with FDA's Closer to Zero action plan, we note that specifications for heavy metals should reflect the amounts determined in the analyses of representative batches and be kept as low as possible.**

   Response:

   Please refer to Annexure 2 for the heavy metal analysis of maltogenic alpha amylase according to the AOAC 2015.01 method.

   Revised specification for heavy metals for maltogenic alpha amylase from *E. coli* BLASC is provided

[1] Safety evaluation of the food enzyme maltogenic amylase from a genetically modified *Bacillus subtilis* (strain NZYM-SM) (EFSA Journal 2018;16(5):5171)

below:

Heavy Metal	Limit of Quantification LOQ (mg/Kg)	Heavy metal concentration in maltogenic alpha amylase batches			Specification (mg/Kg)
		Batch No. 012244	Batch No. 012246	Batch No. 012248	
Arsenic	0.1	0.14	0.19	0.15	Not more than 0.5 ppm
Cadmium	0.1	<0.1	<0.1	<0.1	Not more than 0.25 ppm
Lead	0.1	<0.1	<0.1	<0.1	Not more than 0.5 ppm
Mercury	0.025	<0.025	<0.025	<0.025	Not more than 0.1 ppm

4. **Please provide information on the assumptions for solids and liquids used for the Budget method estimation of the theoretical maximum daily intake presented on page 34 of the notice.**

Response:

The information on assumptions for solids and liquids used for estimating theoretical maximum daily intake was taken from the budget method described in chapter 6 of Environmental Health Criteria (EHC) 240. EHC 240 refers to commonly used default proportions of 12.5% for foods and 25% for non-milk beverages. (WHO EHC Chapter 6, 2009)[2].

5. **Please confirm that you have conducted a comprehensive literature search pertaining to the safety of maltogenic alpha-amylase and have not identified information that would contradict your GRAS conclusion. Please also provide the date (month/year) of your most recent literature search.**

Response:

We confirm that we have conducted a comprehensive literature search pertaining to the safety of maltogenic alpha amylase and have not identified information that would contradicted our GRAS conclusion. The most recent search was conducted for Maltogenic alpha amylase from *E coli* BLASC prior to its submission to the USFDA in May 2022.

6. **In your bioinformatics analysis, you demonstrate that maltogenic alpha-amylase shares >35% sequence identity with the fungal allergen, Asp o 21, across a window of 80 amino acids. We note that Asp o 21 is not a registered food allergen; however, there are published reports of food allergy from consumption of alpha-amylase[3]. These cases are thought to be due to occupational sensitization. Please confirm that Advanced Enzymes concludes that the identified allergen is not expected to result in allergenicity concerns from oral exposure.**

Response:

Advanced enzymes confirm that the identified allergen Asp o 21 is not expected to result in the allergenicity concerns from the oral exposure.

---

[2] Principles and methods for the risk assessment of chemicals in food (Environmental Health Criteria, No. 240). Rome: Food and Agriculture Organization of the United Nations; Geneva: World Health Organization, International Programme on Chemical Safety; 2009: Chapter 6: Dietary exposure assessment of chemicals in food.

[3] Moreno-Ancillo A. et al., (2004). Bread eating induced oral angioedema due to alpha-amylase allergy. *J Investig Allergol Clin Immunol.*, 14, 346-7.

Overbey, Katie

From: Madhu Soni <sonim@bellsouth.net>
Sent: Tuesday, October 24, 2023 8:27 AM
To: Overbey, Katie
Subject: [EXTERNAL] RE: GRN 1097 - Additional FDA Questions

Dear Dr. Overbey,

Please see below our responses to your queries:

FDA Query 1: Based on calculations on page 34 of the notice, the notifier states that for solid foods, the intake of baking goods is 12.5 g/kg bw/day. The Budget method is based on daily energy intake of 100kcal/kg bw/day, which is equivalent to 50 g solid food/kg bw/day. Please confirm if the assumption for consumption of baked goods is 25% (based on the calculations) or 12.5% based on the response to question 4 in the amendment dated 9/11/2023

Response: 1.

The Budget Method is based on the following assumed consumption of important foodstuffs and beverages (for less important foodstuffs, e.g. snacks, lower consumption levels are assumed) presented in the table below:

Assumed consumption of important foodstuffs and beverages - Budget Method

Average consumption over the course of a lifetime/kg body weight/day	Total solid food (kg)	Total non-milk beverages (l)	Processed food (50% of total solid food) (kg)	Soft drinks (25% of total beverages) (l)
	0.025	0.1	**0.0125**	**0.025**

We confirm that the assumption for consumption of solid food is 25%.

50% of food consumed are processed food. Therefore, value considered for the calculation is 0.0125. [1]

FDA Query 2: Please confirm that in the dietary exposure estimate for maltogenic alpha-amylase, it is assumed that all of the maltogenic alpha-amylase will be active and remain in the final food.

Response: 2.

We confirm that, in estimating dietary exposure, we made the worst-case assumption that all of the active maltogenic alpha amylase would still be present in the final food.

However, in practice/in real situation, it does not exert enzymatic activity in the final food due to denaturation of the enzyme during processing.

If you have any questions or need clarification, please let us know. Thank you

Best regards
Madhu

[1] Douglass JS, Barraj 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

From: Overbey, Katie [mailto:Katie.Overbey@fda.hhs.gov]
Sent: Monday, October 16, 2023 11:22 AM
To: Madhu Soni <sonim@bellsouth.net>
Subject: GRN 1097 - Additional FDA Questions

Dear Dr. Soni,
During our review of GRAS Notice 1097, we identified additional questions that need to be addressed. Please find the questions below.

1. Based on calculations on page 34 of the notice, the notifier states that for solid foods, the intake of baking goods is 12.5 g/kg bw/day. The Budget method is based on daily energy intake of 100kcal/kg bw/day, which is equivalent to 50 g solid food/kg bw/day. Please confirm if the assumption for consumption of baked goods is 25% (based on the calculations) or 12.5% based on the response to question 4 in the amendment dated 9/11/2023.
2. Please confirm that in the dietary exposure estimate for maltogenic alpha-amylase, it is assumed that all of the maltogenic alpha-amylase will be active and remain in the final food.

You may reply directly to this email with your responses or send them as a PDF. Please ensure that your responses do not contain confidential business information and please do not submit a revised version of the GRAS notice.

We respectfully request a response to these questions within 10 business days. If you are unable to complete the response within that time frame, please contact me to discuss further options.

Thank you in advance for your attention to our comments.

Best,
Katie

Katie Overbey, Ph.D., M.S (she/her/hers)
Regulatory Review Scientist

Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
Tel: 240-402-7536
katie.overbey@fda.hhs.gov