PROCESSING AID APPLICATION

Food Standards Australia New Zealand

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

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
CCI	Confidential Commercial Information
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
ECCB	Exclusive Capturable Commercial Benefit
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	Liter
LD_{50}	Median Lethal Dose
MANU	Maltogenic Amylase Unit
MCS	Multiple Cloning site
mg	Milligram
ml	Milliliter
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
Mmol	Milli Mole

INTRODUCTION

The present dossier describes an enzyme maltogenic alpha amylase from *Geobacillus stearothermophilus* produced by a recombinant *Escherichia coli* strain and used in food processing. The enzyme is produced by the submerged fermentation. The processing aid maltogenic alpha amylase is marketed by M/s Advanced Enzymes with trade names such as SEBake Fresh 1.5P, SEBake Fresh 10P, SEBake Fresh 20P, SEBake Fresh 50P, SEBake Fresh 100P.

Maltogenic alpha amylase (IUBMB 3.2.1.133) catalyses the hydrolysis of (1→4)-alpha-D-glucosidic linkages in polysaccharides such as starch and primarily remove successive alpha-maltose residues from the non-reducing ends of the hexose chains.

Maltogenic alpha amylase is used as a processing aid in baking, brewing and starch processing.

Detailed descriptions of construction of the genetically modified *E. coli* strain, designated as *E. coli* BLASC (the production microorganism); the enzyme production process; product specifications; applications of the enzyme in food processes; and finally the safety evaluation of the processing aid including the toxicology studies have been provided in different sections in this dossier. The information provided is evaluated to confirm the safety of the enzyme for its intended use.

The documentation has been elaborated according to the Application Handbook from Food Standards Australia New Zealand as of July 1st 2019, applied as relevant for an enzyme application, i.e. outlining the following section:

• Section 3.1.1 – General requirements

• Section 3.3.2 – Processing aids, subsections A, B, C, D, E, and F

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

1. GENERAL INFORMATION

1.1 Applicant details

Advanced Enzymes Technologies Ltd. submits this processing aid application to amend Schedule 18 of the Australia New Zealand Food Standards Code.

NAME AND ADDRESS OF APPLICANT

[redacted]

MANUFACTURER

[redacted]

PERSON RESPONSIBLE FOR THE DOSSIER

[redacted]

NATURE OF APPLICANT'S BUSINESS

Advanced Enzymes is a manufacturer and marketer of enzymes and probiotics.

1.2 Purpose of the application

This application is submitted to provide for amendment of 'schedule 18- Processing aids' of Australia New Zealand Food Standards Code, to include the processing aid maltogenic alpha

amylase produced by a genetically modified *Escherichia coli* BLASC as permitted source for the maltogenic alpha amylase.

1.3 Justification for the application

THE NEED FOR THE PROPOSED CHANGE

'Schedule 18—Processing aids' of Australia New Zealand Food Standards Code, contains a list of permitted enzymes of microbial origin. The processing aid 'maltogenic alpha amylase' produced by a genetically modified strain of *Bacillus subtilis,* expressing the gene from *Geobacillus stearothermophilus,* has been evaluated and included in the schedule 18. The maltogenic alpha amylase enzyme produced by recombinant *E. coli* BLASC, expressing the similar gene from *Geobacillus stearothermophilus* is, however, not evaluated and included as an approved source in the schedule 18 of standard 1.3.3- Permitted enzymes of microbial origin.

M/s Advanced Enzyme Technologies Ltd uses the recombinant strain *E. coli* BLASC to produce the similar processing aid, the maltogenic alpha amylase. Advanced Enzymes Technologies Ltd. is requesting to add this source organism in the schedule 18.

THE ADVANTAGES OF THE PROPOSED CHANGE OVER THE *STATUS QUO*

Maltogenic alpha amylase is used as a processing aid in baking, brewing and in starch processing applications.

The advantages of the proposed change over the *status quo* can be listed as below

- The processing aid is very well accepted in food processing industries and currently there are limited suppliers. The proposed change will ensure easy availability of the processing aid.
- The change in *status quo* will benefit the food processors in Australia and New Zealand as the manufacturer M/s Advanced Enzymes extends strong application research supports to its' customers.

Benefits of the use of the maltogenic alpha amylase in certain food processes are listed below:

Baking

- Formation of oligosaccharides with different degrees of polymerization (DP) from amylopectin. (Refer to Annex M1 for action of Maltogenic amylase on amylopectin)
- Imparting anti-staling benefits in baked products such as breads, thereby extending shelf life. Enhancing crumb softness, resilience, loaf volume and texture, resulting in superior quality products (Refer to Annex M2 for effect of maltogenic amylase on the properties of the bread).

Starch processing for production of maltose/ glucose syrup

- Improved yields of maltose/ glucose in reaction.
- Reduced risk of microbial contamination due to high saccharification temperature.

- Reduction in processing time.
- Improved filterability owing to reduction of long chain dextrins.

(Annex M3 describes action of maltogenic amylase on corn starch modification and its advantages)

Maltogenic alpha amylase from *B. stearothermophilus* reduces viscosity of unmalted flour, owing to starch hydrolysis (Brumm et al. 1988). The increased levels of glucose and maltose compared to maltodextrins in starch hydrolysates, have a positive effect on clarity and filterability (Slominska, 1989) of hydrolysates. The maltogenic amylase hydrolyses starch and produces high amounts of maltose individually and also synergistically works with other enzymes and improves filterability (observation also shared by customers). The in-house data from brewing trials (Annex M4) also shows an increased filterability of wort in the brewing process. Increased filterability and a higher yield of maltose in various application trials results in decreased production time.

Brewing

- Improved yield due to release of high amount of maltose sugar.
- Decreased production time.
- Hydrolysing starch from various grains into maltose.

(Refer to Annex M4 for action of Maltogenic amylase in brewing process and decrease in production time and refer to Annex M5 for action of maltogenic amylase on the hydrolysis of starch from various grains)

1.4 Information to support the application

DATA REQUIREMENT

Sections 2 - 7 of this application contain detailed data that establishes the quality, efficacy, and safety of the processing aid maltogenic alpha amylase derived from *E. coli* BLASC, under the proposed conditions of use in Australia and New Zealand, as presented in accordance with the information requirements listed in Section 3.3.2 (Processing Aids) of the Food Standards Australia New Zealand (FSANZ) Application Handbook (FSANZ, 2019). The data pertaining to the maltogenic alpha amylase derived from *E. coli* BLASC presented in this application is representative of the commercial product for which approval is being sought.

1.5 Regulatory impact information

COST AND BENEFITS OF THE APPLICATION

The application is not likely to place costs or regulatory restrictions on industry or consumers. Maltogenic alpha amylase is already included in the Schedule 18 as a processing aid. This application is to include another microorganism, i.e., a recombinant *E. coli* strain BLASC as a production host in the Schedule 18 for the same processing aid. For government, the burden is limited to necessary activities for an updation of Schedule 18—Processing aids.

IMPACT ON INTERNATIONAL TRADE

The application is not likely to cause an impact on international trade. The processing aid is already in use in Australia and New Zealand. The present application will add another supplier for the processing aid.

1.6 Assessment procedure

The application is for a new source organism for an existing enzyme in the Code. Therefore, the appropriate assessment procedure for the application is considered to be a "General Procedure, Level 1".

1.7 Confidential commercial information (CCI)

Detailed information on the genetic modifications and characteristics of the genetically modified production strain is provided in Annex I conf. A summary of this information is given in section 6.

The identity and depository information of the production organism are provided in Annex F Conf. and Annex E Conf. A summary of this information is given in section 5.1.

The formal request for treatment of selected parts of Annexes as confidential commercial information (CCI) is provided in Annex J.

1.8 Other confidential information

Apart from the Annexes E Conf., F Conf. and I Conf., identified as confidential commercial information (CCI), no other information is requested to be treated as confidential.

1.9 Exclusive capturable commercial benefit (ECCB)

This application is not expected to confer an Exclusive Capturable Commercial Benefit for AETL or to any other individual company.

1.10 International and other national standards

INTERNATIONAL STANDARDS

Use of enzymes as processing aids for food production is not restricted by any Codex Alimentarius Commission (Codex) Standards.

OTHER NATIONAL STANDARDS OR REGULATIONS

To our knowledge use of enzymes as processing aids for food production is in general not restricted by standards or regulations in other countries. Application for use of the maltogenic alpha amylase from the strain *E. coli* BLASC has already been evaluated by the European Food Safety Authority. Maltogenic alpha amylase from *Geobacillus stearothermophilus* in general has been evaluated by various regulators and approved for use as a processing aid (Section 4.1)

1.11 Statutory declaration

The Statutory Declaration is provided as Annex K along with the submission.

1.12 Checklist

This application concerns an enzyme product intended to be used as a processing aid. Therefore, the relevant documentation according to the Application Handbook from Food Standards Australia New Zealand as of July 1, 2019 has been provided.

The checklists for General requirements as well as the Processing aids part are appended below.

CHECKLIST

			General requirements (3.1.1)
Check	**Page No.**	**Relevant sections of dossier**	**Mandatory requirements**
☑	–	–	A Form of application ☑*Application in English* ☑*Executive Summary (separated from main application electronically)* ☑*Relevant sections of Part 3 clearly identified* ☑*Pages sequentially numbered* ☑*Electronic copy (searchable)*
☑	7	1.1	B Applicant details
☑	7	1.2	C Purpose of the application
☑	8 9	1.3 1.4	D Justification for the application ☑*Regulatory impact information* ☑*Impact on international trade*
☑	9	1.5	E Information to support the application ☑ *Data requirements*
☑	10	1.6	F Assessment procedure ☑*General* ☐ *Major* ☐ *Minor* ☐ *High level health claim variation*
☑	10	1.7	G Confidential commercial information ☑*CCI material separated from other application material* ☑*Formal request including reasons* ☑*Non-confidential summary provided*
☑	10	1.8	H Other confidential information ☑*Confidential material separated from other application material* ☑*Formal request including reasons*
☑	10	1.9	I Exclusive Capturable Commercial Benefit ☑*Justification provided*
☑	10	1.10	J International and other national standards ☑*International standards* ☑*Other national standards*
☑	11	1.11	K Statutory Declaration
☑	11-12	1.12	L Checklist/s provided with application ☑*3.1.1 Checklist* ☑*All page number references from application included* ☑*Any other relevant checklists for Chapters 3.2–3.7*

			Processing aids (3.3.2)
Check	**Page No.**	**Relevant sections of dossier**	**Mandatory requirements**
☑	14	2.1	A.1 Type of processing aid
☑	14	2.2	A.2 Identification information
☑	16	2.3	A.3 Chemical and physical properties
☑	20	2.4	A.4 Manufacturing process
☑	25	2.5	A.5 Specification information
☑	27	2.6	A.6 Analytical method for detection
☐	28	3- Not relevant	B.1 Industrial use information (chemical only)
☐	_	Not relevant	B.2 Information on use in other countries (chemical only)
☐	_	Not relevant	B.3 Toxicokinetics and metabolism information (chemical only)
☐	_	Not relevant	B.4 Toxicity information (chemical only)
☐	_	Not relevant	B.5 Safety assessments from international agencies (chemical only)
☑	29	4.1	C.1 Information on enzyme use on other countries (enzyme only)
☑	29	4.2	C.2 Toxicity information of enzyme (enzyme only)
☑	32	4.3	C.3. Allergenicity information of enzyme (enzyme only)
☑	34	4.4	C.4. Overseas safety Assessment Reports
☑	35	5.1	D.1 Information on source organism (enzyme from microorganism only)
☑	36	5.2	D.2 Pathogenicity and toxicity of source microorganism (enzyme from microorganism only)
☑	37	5.3	D.3 Genetic stability of source organism (enzyme from microorganism only)
☑	38	6.1	E.1 Nature of genetic modification of source organism (enzyme from GM source microorganism)
☑	42	7.1	F.1 List of foods likely to contain the processing aid
☑	46	7.2	F.2 Anticipated residue levels in foods
☑	49	7.3	F.3 Information on likely level of consumption
☑	49	7.4	F.4 Percentage of food group to use processing aid
☑	49	7.5	F.5 Information on residues in foods in other countries (if available)
☑	49	7.6	F.6 Where consumption has changed, information on likely consumption

2. TECHNICAL INFORMATION ON THE PROCESSING AID

2.1 Information on the type of processing aid

The maltogenic alpha amylase is an enzyme of microbial origin falls into the category of processing aids as described in Schedule-18 of standard 1.3.3 – Permitted enzymes of microbial origin.

The maltogenic alpha amylase is being used in the food industry as a processing aid for processing of starch containing raw materials. It catalyses the hydrolysis (1→4)-alpha-D-glucosidic linkages in polysaccharides.

Maltogenic alpha amylase is used in the following food manufacturing processes:

- Baking
- Starch processing
- Brewing

The highest recommended dose in all processes listed above is up to 33.90 mg TOS /kg of maltogenic alpha amylase (Enzyme product activity is approx. 100,000 MAN U/g) required to convert starch dry matter. (Refer to Section 7.2)

The *Quantum Satis* (QS) principle is generally used in case of food enzyme preparations. The average dosage of the enzyme depends on the application, the type and quality of the raw materials used, and the process conditions. A further description of the enzyme in these food technology applications is provided in subsequent sections.

2.2 Information on the identity of processing aid

2.2.1 *SCIENTIFIC NAME, TAXONOMY AND OTHER NAMES*

Name of the enzyme protein:	Maltogenic alpha amylase
Synonyms:	glucan 1,4-α-maltohydrolase
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
Marketing / Trade name:	Commercial preparations are known as SEBake Fresh 1.5P, SEBake Fresh 10P, SEBake Fresh 20P, SEBake Fresh 50P, SEBake Fresh 100P.

Microbial source:	Maltogenic alpha amylase is produced by submerged fermentation of *Escherichia coli* BLASC carrying maltogenic alpha amylase gene from *Geobacillus stearothermophilus.*

The enzyme protein is not subjected to a post-translational modification, and is not protein-engineered.

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

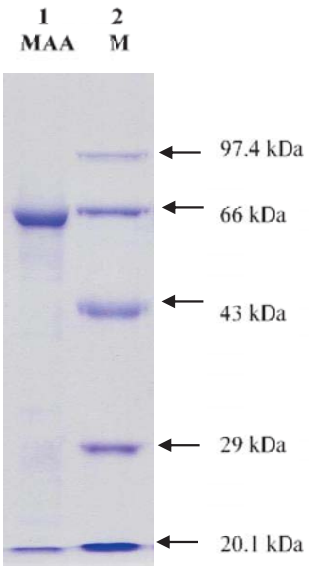
Maltogenic alpha amylase from *E. coli* BLASC is comprised of 686 amino acids having a following sequence:

SSSASVKGDVIYQIIIDRFYDGDTTNNNPAKSYGLYDPTKSKWKMYWGGDLEGVRQ
KLPYLKQLGVTTIWLSPVLDNLDTLAGTDNTGYHGYWTRDFKQIEEHFGNWTTFDT
LVNDAHQNGIKVIVDFVPNHSTPFKANDSTFAEGGALYNNGTYMGNYFDDATKGYF
HHNGDISNWDDRYEAQWKNFTDPAGFSLADLSQENGTIAQYLTDAAVQLVAHGAD
GLRIDAVKHFNSGFSKSLADKLYQKKDIFLVGEWYGDDPGTANHLEKVRYANNSGV
NVLDFDLNTVIRNVFGTFTQTMYDLNNMVNQTGNEYKYKENLITFIDNHDMSRFLS
VNSNKANLHQALAFILTSRGTPSIYYGTEQYMAGGNDPYNRGMMPAFDTTTTAFKE
VSTLAGLRRNNAAIQYGTTTQRWINNDVYIYERKFFNDVVLVAINRNTQSSYSISGLQ
TALPNGSYADYLSGLLGGNGISVSNGSVASFTLAPGAVSVWQYSTSASAPQIGSVAP
NMGIPGNVVTIDGKGFGTTQGTVTFGGVTATVKSWTSNRIEVYVPNMAAGLTDVKV
TAGGVSSNLYSYNILSGTQTSVVFTVKSAPPTNLGDKIYLTGNIPELGNWSTDTSGAV
NNAQGPLLAPNYPDWFYVFSVPAGKTIQFKFFIKRADGTIQWENGSNHVATTPTGAT
GNITVTWQN

Molecular weight of the maltogenic alpha amylase from *E. coli* BLASC was determined as 62 kDa following SDS-PAGE, which is in agreement with the molecular weight obtained with gel permeation chromatography method, indicating that the enzyme is a monomer.

The maltogenic alpha amylase sample was analysed on a 10% SDS-PAGE gel. The zymogram analysis indicated that the protein at 62 kDa, has maltogenic alpha amylase activity. Schiff's staining of the SDS-PAGE gel showed that this protein is not glycosylated.

SDS PAGE analysis of maltogenic alpha amylase derived from *E. coli* BLASC.



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

2.3 Information on the chemical and physical properties of the processing aid

The maltogenic alpha amylase concentrate is derived from the cell biomass collected at the end of the fermentation. There is no detectable amount of other enzyme activities. The enzyme concentrate is spray dried and if required, it suitably diluted with food grade additive such as maltodextrin.

2.3.1 *ENZYME ACTIVITY*

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

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 alpha amylase from *E. coli* BLASC described in this dossier does not possess any detectable enzymatic side activities, which might cause adverse effects.

2.3.1.1 Information on the pH and temperature optima of the food enzyme

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

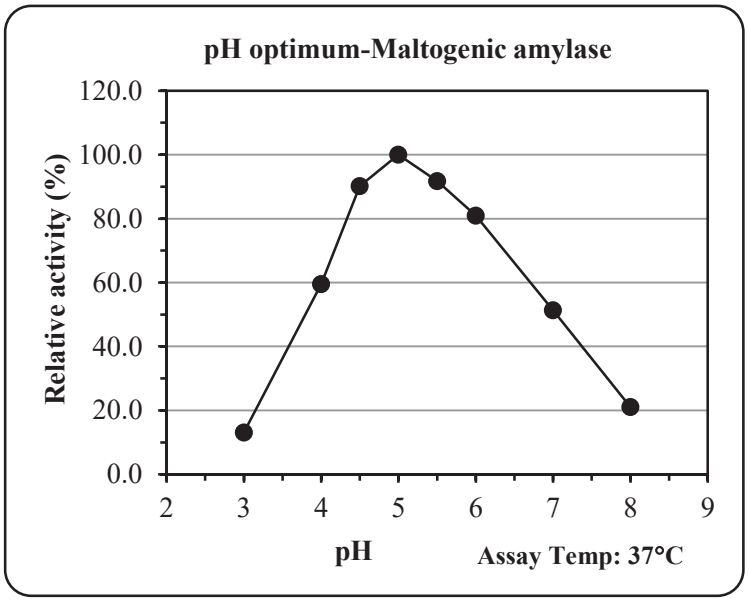


Fig. 2: pH optimum of the maltogenic alpha amylase

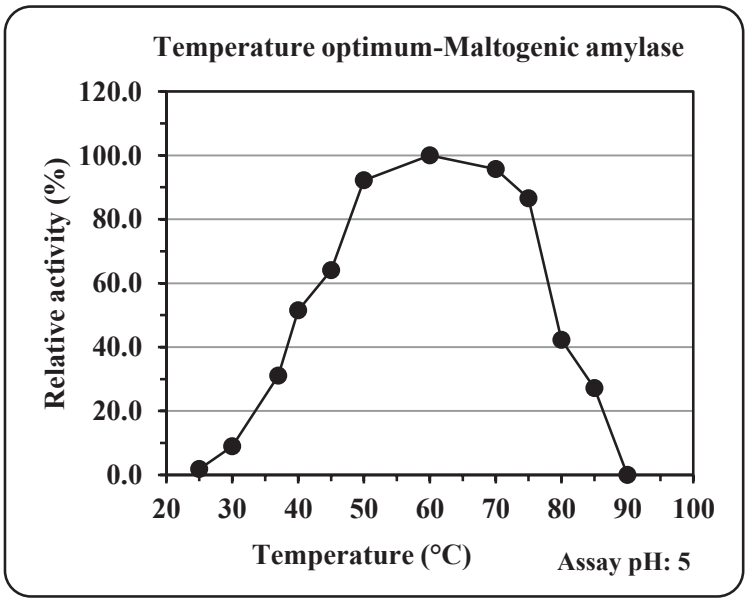


Fig 3: Temperature optimum of the maltogenic alpha amylase

The maltogenic alpha amylase exhibited activity between pH 3.0 to 8.0 (fig. 2) and temperature 25°C to 90°C, with pH 5.0 and temperature 60°C as optimum for activity (fig. 3).

2.3.1.2 Information on the stability of the food enzyme

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

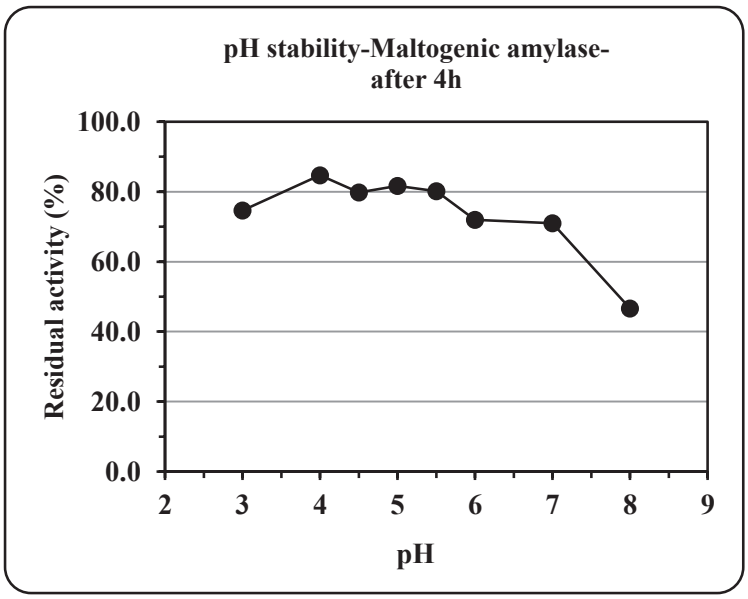


Fig.4: pH stability of the maltogenic alpha amylase

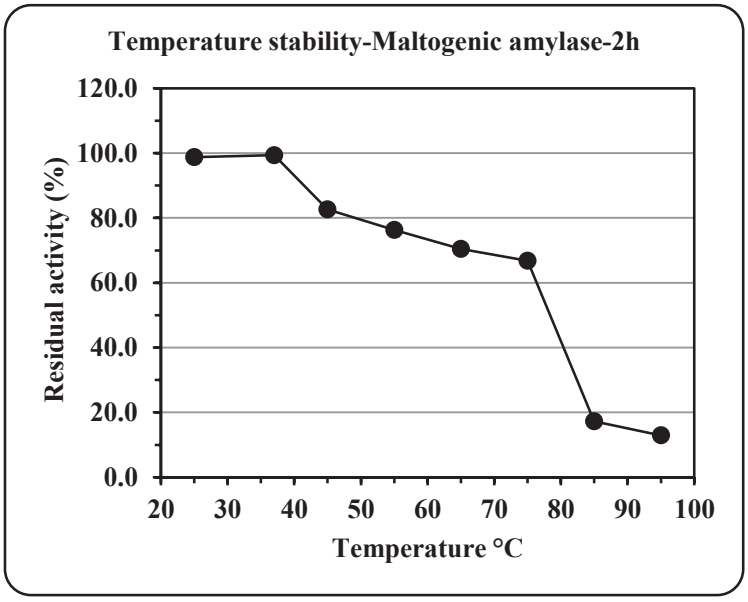


Fig. 5: Temperature stability of the maltogenic alpha amylase

The maltogenic alpha amylase from *E. coli* BLASC was stable between pH 4.0 to 5.5 for 4 hours (fig. 4). 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. 5).

2.3.2 *INTERACTION OF ENZYME WITH DIFFERENT FOODS*

Maltogenic alpha amylase performs its technological function during food processing. It does not perform any technological function in the final food and have no enzymatic activity in the final food. This can be attributed to a combination of 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.

2.3.3 *NUTRITIONAL IMPLICATION*

Maltogenic alpha amylase is a protein and any residual amounts remaining in food consumed would accordingly have the same nutritional value. However, the use levels of maltogenic alpha amylase are very low, and use of this preparation would not have any nutritional significance.

2.4 Manufacturing process

2.4.1 *OVERVIEW*

Maltogenic alpha amylase is produced following a fed batch fermentation process. Fermentation is carried out in accordance with 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.

Fermentation is a well-known process that occurs in food and has been used for the production of food enzymes for decades. The typical fermentation batch size for the production of the maltogenic amylase ranges from 100 L to 50,000 L, preferably 15,000 to 18,000 L. The frequency of production is planned depending on the market demand.

As shown below, the key steps for production of the maltogenic alpha amylase are fermentation, recovery, formulation, and packaging. The process is illustrated in Fig 6.

2.4.2 *FERMENTATION*

2.4.2.1 Raw materials

Materials used in the fermentation process (inoculum, seed, and main fermentation) are all food-grade substances, approved for use. There are no ingredients based on milk, soy, or any of the top eight allergens (FALCPA, 2004; ETA 2005).

Potable water
A carbon source
A nitrogen source
Salts
Vitamins (as a part of complex fermentation materials)
pH adjustment agents
Foam control agent (at ≤0.1%)
Inducer - for induction of maltogenic alpha amylase synthesis

2.4.2.2 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.

2.4.2.3 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.

2.4.2.4 Main fermentation

The enzyme production takes place at this stage. The fermentation is operated in a 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 in order to induce the enzyme production, enzyme 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 considered complete.

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

2.4.3 *RECOVERY*

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

During fermentation, the enzyme protein maltogenic alpha amylase is produced intracellularly and retained inside the cells of production microorganism. 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

2.4.3.1 Primary Separation

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

2.4.3.2 Concentration and centrifugation

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

controlled during this step. The filtered liquid is passed through a high-speed centrifuge to separate and remove other insoluble and partially soluble impurities.

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

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

2.4.3.4 Spray Drying

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

2.4.4 *FORMULATION AND PACKAGING*

Food enzyme maltogenic alpha amylase is sold as powder preparations having varying maltogenic alpha amylase activities.

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

2.4.5 *INFORMATION ON RECOMBINANT DNA TECHNIQUES USED IN THE PREPARATION OF GENETICALLY MODIFIED ORGANISM*

Refer to Section no. 6 for the information on recombinant DNA techniques used in the preparation of genetically modified organism used for the production of maltogenic alpha amylase.

2.4.6 *ALLERGENICITY OF ENZYME PREPARATIONS*

Materials used in the fermentation process (inoculum, seed, and main fermentation) of maltogenic alpha amylase are food-grade substances. There are no ingredients based on milk, soy, or any of the top eight allergens used during fermentation process. (FALCPA, 2004).

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

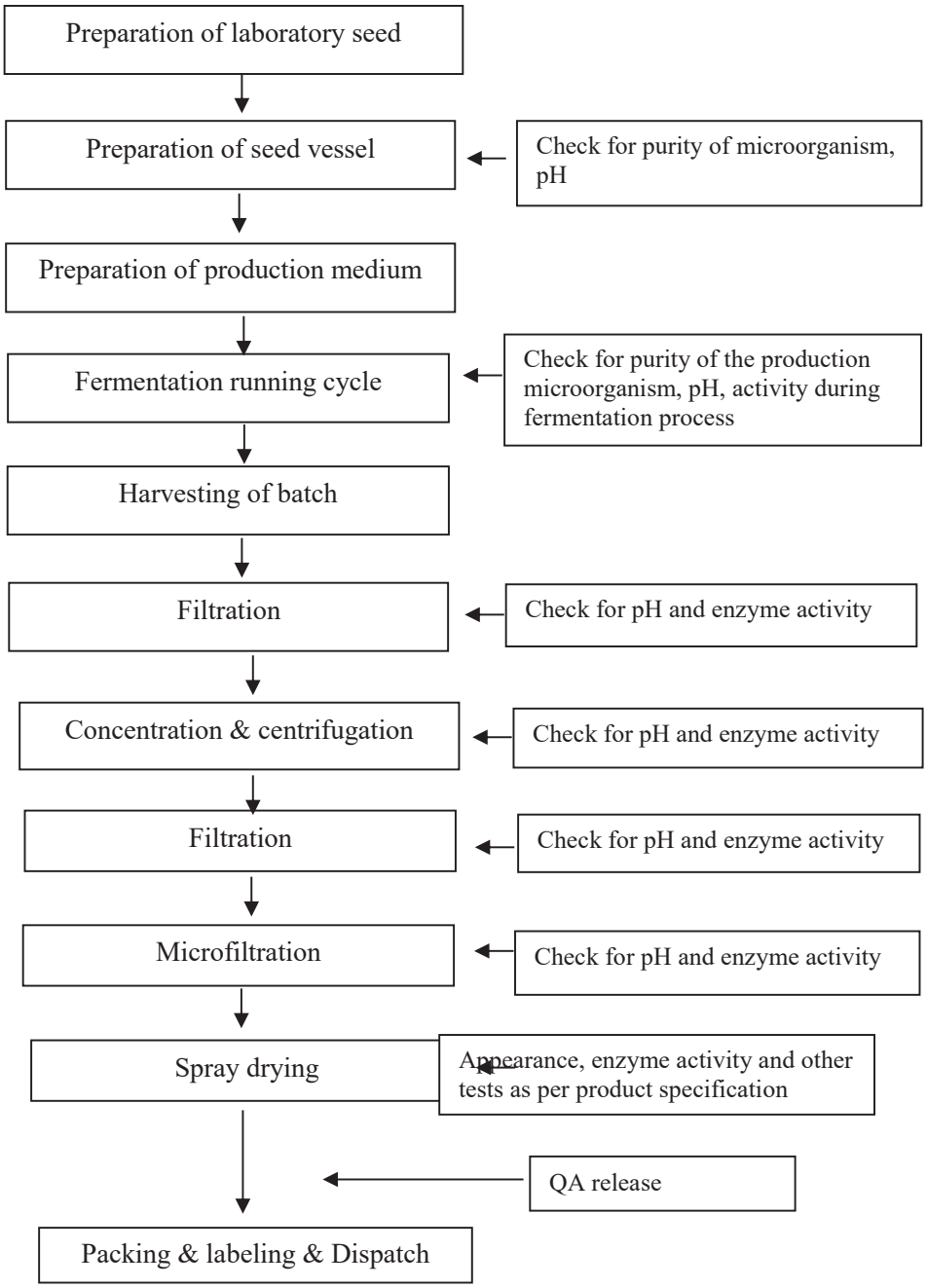


Fig.6: Manufacturing process of the maltogenic alpha amylase from *E. coli* BLASC

2.4.7 *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, 2006b), 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 Pharmacopoeial method (USP,EP and JP) and IP
Total coliforms	Not more than 30 per gram	Harmonized Pharmacopoeial method (USP,EP and JP) and IP
Escherichia coli	Absent in 25 g of sample	Harmonized Pharmacopoeial method (USP,EP and JP) and IP
Antimicrobial activity	Not detected	JECFA 2003 (FNP 52, Add. 11)

The specifications of the maltogenic alpha amylase produced by *E. coli* BLASC are described in Section 2.5.

The proof that the maltogenic alpha amylase from *E. coli* BLASC complies with these specifications is shown by the analyses on three production batches. Refer to Annex B for analyses of heavy metals; compliance with the microbial specification and antimicrobial activity is presented in the signed certificate of analysis for the individual batches. (Annex N) (Antimicrobial analysis is also provided in section 4.2.4 of the dossier). Annex A1 provides information covering microbial analytical methods.

The Bacterial strains do not produce mycotoxins, however as an abundance of caution, the product maltogenic alpha amylase from *E. coli* BLASC is tested for presence of mycotoxins, which could be contributed by contaminated processing material. Refer to Annex C for analyses of mycotoxin[1].

[1] Refer JECFA specifications [FAO/WHO (2006b)], Compendium of food additive specification Monograph 3,Page 64. http://www.fao.org/3/a-a0675e.pdf

2.5 Specifications for identity and purity

2.5.1 *PRODUCT SPECIFICATIONS*

Specifications for maltogenic alpha amylase preparation have been established by Advanced Enzyme Technologies Ltd. and are summarized in Table 1. All methods are standard and provided in Annexes A1 and A2.

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

Product specification	Advanced Enzyme Technologies Ltd.	
	Limits	Reference Method
Maltogenic alpha 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%	Harmonized Pharmacopoeial method (USP,EP and JP) and IP
Solubility	Soluble in water	Harmonized Pharmacopoeial 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 Pharmacopoeial method (USP,EP and JP) and IP
Total coliform	Not more than 30 cfu/g	Harmonized Pharmacopoeial method (USP,EP and JP) and IP
E. coli /25g	Negative by test	Harmonized Pharmacopoeial method (USP,EP and JP) and IP
Salmonella spp. /25g	Negative by test	Harmonized Pharmacopoeial method (USP,EP and JP) and IP
Antimicrobial activity	Absent by test	JECFA 2003 (FNP 52, Add. 11)

2.5.2 *COMPOSITIONAL VARIABILITY (RELATIVE PURITY)*

Commercial enzymes, whether used in the production of food, feed or for technological purposes, are biological isolates of variable composition. Apart from the enzyme protein in question, microbial food enzymes also contain some substances derived from the producing micro-organism and the fermentation medium. These constituents consist of organic material and inorganic salts. As established by JECFA (FAO/WHO, 2006a, 2006b), the percentages of these organic materials are summarized and expressed as Total Organic Solids (TOS)[2].

2.5.2.1 Quantitative Composition

Relative purity of maltogenic alpha 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 alpha amylase from *E. coli* BLASC

Batch no	031434[3]	071404	071405	Mean enzyme activity
Ash (%)	8.86	8.21	7.75	-
Water (%)	7.35	6.97	6.68	-
TOS (%)	83.79	84.82	85.57	-
Maltogenic alpha amylase activity (MAN U/g)	144255	153647	158521	152141
U/mg TOS	172.16	181.14	185.25	-
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.				

The enzyme activity, ash, and dry matter content were determined using standard methods. These standardized methods are provided in Annexes A1 and A2.

[2] The TOS value is an internationally accepted method to describe the chemical composition of commercial food enzymes. The ratio between the enzyme activity and TOS is an indication of the relative purity of the enzyme.
[3] The batch used for toxicity studies adheres to JECFA specifications for food enzyme(s).

2.5.2.2 Data on batch to batch variability for relevant parameters

Three batches of maltogenic alpha amylase from *E. coli* BLASC were analysed and the results compared with the food-grade specifications. The methods of analysis used to determine purity are mentioned in Annexes A1 and A2. As shown in Table 3, all tested batches complied with the product specifications, demonstrating suitability of the production process.

Maltogenic alpha amylase enzyme preparation does not contain any known allergens.

Table 3: Variability in production batches of the maltogenic alpha amylase from *E. coli* BLASC

Parameter	Specification	Batch		
		031434	071404	071405
Maltogenic alpha amylase Activity	Not less than 100,000 MAN U/g	144255 MAN U/g	153647 MANU/g	158521 MAN U/g
Description	Light brown to brown colored powder with characteristic odor	Light brown colored powder with characteristic odor	Light brown colored powder with characteristic odor	Light brown colored powder with characteristic odor
Solubility	Soluble in water	Soluble in water	Soluble in water	Soluble in water
Moisture/Loss on drying (%)	Not more than 10.0%	7.35%	6.97 %	6.68 %
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	300 cfu/g	450 cfu/g	200 cfu/g
Total Coliform	Not more than 30 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
E. coli	Absent in 25g	Absent in 25g	Absent in 25g	Absent in 25g
Salmonella spp.	Absent in 25g	Absent in 25g	Absent in 25g	Absent in 25g
Antimicrobial activity	Absent by test	Absent by test: Complies	Absent by test: Complies	Absent by test: Complies

2.6 Analytical method for detection

Not applicable for enzymatic processing aid as mentioned in section A.6 of 3.3.2- Processing Aid of Application Handbook (2019).

Maltogenic alpha amylase enzyme preparation is used in the food industry as a processing aid.

3. Information related to the safety of a chemical processing aid

Not applicable for enzymatic processing aid as mentioned in section B of 3.3.2- Processing Aid of Application Handbook (2019).

4 INFORMATION RELATED TO THE SAFETY OF AN ENZYME PROCESSING AID

4.1. General information on the use of the enzyme as a food processing aid in other countries

Maltogenic alpha amylase has a long history of use in food processing. Foods processed with maltogenic alpha amylase are known to be consumed safely by the general human population. There is precedence of regulatory recognition of maltogenic alpha amylase. Following summarizes regulatory recognition for maltogenic alpha 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 from *Geobacillus stearothermophilus*) as a processing aid under Australia New Zealand Food Standards Code - Standard 1.3.3.
- Legifrance 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)
- USFDA has issued no question 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)
- 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] also evaluated by EFSA and no safety concerns were reported.

4.2 Information on the potential toxicity of the enzyme processing aid

4.2.1 *INFORMATION ON THE ENZYME'S PRIOR HISTORY OF HUMAN CONSUMPTION AND/OR ITS SIMILARITY TO PROTEINS WITH A HISTORY OF SAFE HUMAN CONSUMPTION*

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 J.E.C. 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 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 J.E.C. 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.

4.2.2 *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 2.2.2 was used as input for the search.

The maltogenic alpha amylase protein did not show homology with known toxin protein sequences described in database sequences.

Furthermore, safety studies as described below were performed on a representative batch (031434) that was produced according to the description given in section 2.4, omitting stabilization and standardization. A summary of the safety studies is enclosed in series of Annex G1-G4.

4.2.3 *SAFETY OF MALTOGENIC ALPHA AMYLASE – ORAL TOXICITY AND GENOTOXICITY*

Maltogenic alpha amylase from *Geobacillus stearothermophilus* produced by *E. coli* BLASC (Batch no 031434), has been investigated in a series of systemic and genetic toxicity studies complied with OECD Guidelines and conducted in accordance with the principles of Good Laboratory Practice (GLP) as published by the OECD (ENV/MC/CHEM (98)17).

Sample	:	Maltogenic amylase from GMM *E.coli* (strain BLASC): Batch No. 031434
Activity	:	144,255 MAN U/g
TOS	:	83.79%
NOAEL Dose	:	1000 mg/kg of body weight. i.e. 837.9 mg TOS/ kg body weight/day of Maltogenic amylase from GMM *E.coli* (strain BLASC)

Batch no. 031434 used to conduct the toxicity studies have the same specification with that of final product.

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 (1675.8 mgTOS/kg bw , 288,510 MAN U/ /kg body weight) and observed for 14 days. No indications of toxicity were reported. Based on the results, the estimated LD_{50} for maltogenic alpha amylase from *E. coli* BLASC was greater than 2000 mg /kg bw. (Annex G1)

Repeated-dose 90-day oral toxicity test (OECD Test No. 408, 1998): Four groups of 10 male and 10 female Wistar rats, 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. 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 beginning and end of dosing. At week 13, all animals were assessed for sensory reactivity, grip strength, and motor activity. Feed consumption and body weight were recorded weekly. Blood and urine samples were taken at the end of dosing and after recovery. All animals were subjected to necropsy and weights of kidneys, liver, adrenals, testes, epididymis, uterus, thymus, spleen, brain, ovaries, and heart were recorded. Histological evaluations were performed on all tissues from control and high-dose rats.

There was no mortality and no clinical abnormalities 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 alpha 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 G2).

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 alpha amylase from *E. coli* BLASC is non-mutagenic (Annex G3).

***In vitro* mammalian chromosomal aberration test in human lymphocytes (OECD Test No. 473, 1997):** Cultures of human peripheral blood lymphocytes were exposed to maltogenic alpha 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 alpha amylase from *E. coli* BLASC is non-clastogenic in the presence and absence of microsomal enzymes (Annex G4).

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

4.2.4 *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 (USP2008). Maltogenic alpha amylase showed an absence of antimicrobial activity against all the test microorganisms.

Also, heavy metals and mycotoxins levels were well within the specification and below the detection limits of analytical methods. (Annex B and Annex C)

Further, the maltogenic alpha amylase covered in this dossier and produced by *E coli* BLASC does not contain any detectable amount of recombinant DNA as determined by the absence of amplicon for recombinant gene of maltogenic alpha amylase. This confirmed the absence of recombinant DNA in the product. (Refer to section 6.1.4 for details)

4.3 Information on the potential allergenicity of the enzyme processing aid

4.3.1 *SOURCE OF THE ENZYME PROCESSING AID*

Maltogenic alpha amylase is produced by submerged fermentation of non-pathogenic and nontoxic strain of *E. coli* BLASC carrying maltogenic alpha amylase gene from *Geobacillus stearothermophilus.*

4.3.2 *ANALYSIS OF SIMILARITY BETWEEN THE AMINO ACID SEQUENCE OF THE ENZYME AND THAT OF KNOWN ALLERGENS*

Allergenic potential of the maltogenic alpha 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 analysing 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).

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 alpha amylase sequence (as presented in section 2.2.2) was analysed using FARRP database. Bioinformatics searches showed that maltogenic alpha 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 alpha amylase with the amino acid sequences of known allergen, showed no allergenicity concern. (Refer to Annex L for the details)

4.3.3 *INOFORMATION ON THE STABILITY OF THE ENZYME TO DEGRADATION IN APPROPRIATE GASTRIC AND IF APPLICABLE, INTESTINAL MODEL DIGESTION SYSTEM*

Allergenicity of the maltogenic alpha amylase from *E. coli* BLASC was also 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 alpha 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 alpha amylase can be considered to be a labile protein, as it is digested by pepsin, and therefore is unlikely to be allergenic.

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 miniscule quantities of the enzyme(s) in the final food. Goodman et al (2008) discuss that a high concentration generally equals a higher risk of sensitization, whereas a low level in the final food equals a lower risk. Additionally, it must be noted that the food enzyme protein undergoes denaturation under various conditions of food processing, resulting in loss of its tertiary structure. In general, these alterations in conformation are associated with decrease in the antigenic reactivity in humans. Usually, denatured proteins are much less immunogenic than the corresponding native proteins [Takai et al., 1997; Takai et al., 2000 (as cited in Koyanagi et al. 2010); Valenta, 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 alpha amylase enzyme is readily digested by the pepsin. Bioinformatics analyses does not show any similarity of maltogenic alpha amylase amino acid sequence with known allergen. These observations rule out any allergenicity concern with the maltogenic alpha amylase enzyme protein. (Refer to Annex L for details).

4.4 *SAFETY ASSESSMENT REPORTS PREPARED BY INTERNATIONAL AGENCIES OR OTHER NATIONAL GOVERNMENT AGENCIES, IF AVAILABLE*

European Food Safety Authority has evaluated the maltogenic amylase from *E. coli* BLASC and found no safety concern with the processing aid under the intended conditions of use. (EFSA Journal, 2019) (Annex H)

5 ADDITIONAL INFORMATION RELATED TO THE SAFETY OF AN ENZYME PROCESSING AID DERIVED FROM A MICROORGANISM

5.1 Information on the source microorganism

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

'BLASC' is the designation of the *E. coli* strain used for the production of maltogenic alpha 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 (Annex E conf.).

The host strain *E.coli* SAML27 used for the generation of production strain BLASC is a derivative of *E.coli* BL21(DE3) with the following genotype: F-, ompT, lon, hsdSB (restriction- modification-), gal, dcm, DE3 (lacI, T7polymerase under the control of the PlacUV5 promoter), ccdB+.

The production strain BLASC was characterized following 16S rRNA gene as phylogenetic markers. The primers 16SF27 and 16SR1525 were utilized for amplification and sequencing of 16S rRNA gene from the strain BLASC. The 16S rRNA gene sequence of strain BLASC was identified by BLAST and phylogenetic analysis. The BLAST and phylogenetic analysis of 16S rRNA gene sequence showed 100% homology to *Escherichia coli* BL21 (DE3). (Annex F Conf.)

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 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 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)

Besides *E. coli* BL21, other *Escherichia coli* strains such K-12, B, C and W are frequently used. Processing aid produced by *E coli*, such as Chymosin (EC 3.4.23.4) from *Escherichia*

coli K-12 strain GE81, has been evaluated by FSANZ and included as an approved production host in Schedule 18. *E. coli* B and BL21 cells are mainly used for expression of genes (Bauer et al, 2007). *E. coli* BL21 is the most common industrial host known to produce several recombinant proteins for production of biopharmaceuticals (Kamionka, 2011) and industrial enzymes. Most common therapeutic proteins expressed in *E.coli* are Human insulin (Nilsson et al, 1996), interleukin 2 (IL-2) (Roifman, 1985), etc. Several industrial enzymes have also been produced in *E.coli* viz. Xylanase (Whitehead and Hespell, 1989), betaglucosidase (Ferreira, 2018), cellulases (Amraini et al, 2017), etc. Thus, *E. coli* strains can be considered as safe production organisms for enzymes used in food / feed processing as well as numerous other industrial applications.

The BL-21 strain of *E. coli* has been safely used in the production of alpha-Cyclodextrin glycotransferase. (Maksum et al., 2020) Furthermore, *E. coli* BL- 21 strain has been used as a laboratory organism for over a decade without reported incidents of infection and that 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).

5.2 Information on the pathogenicity and toxicity of the source microorganism

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

The production strain does not produce secondary metabolites of toxicological concern to the human. Mycotoxins and heavy metal contents in the maltogenic alpha amylase produced by *E. coli* BLASC were within the specifications and below the detection limits of the test methods. (Annex B and Annex C). The safety of production organism was further substantiated by lack of toxicity, when the maltogenic alpha amylase from *E. coli* BLASC was tested in standard *in-vitro* and *in-vivo* toxicity model systems (Refer to section 4.2.3 for details).

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 reported 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 was 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 produce 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 representative in alignment process, which generate non-overlapping alignment. This concluded the absence of toxin gene in these strains. Therefore, expressing recombinant protein, especially therapeutic protein, in *E. coli* was considered to be safe against toxin. (Maksum et. al., 2020).

Also, significant number of short-term and sub-chronic 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 production of recombinant proteins further supports the conclusion that *E. coli* BL21(DE3) is 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 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)

5.3 Information on the genetic stability of the source microorganism

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, enables to stabilize the plasmid completely during the fermentation. There is no risk to have the production strain without plasmid, which is due to the post-segregational killing. To demonstrate the genetic stability of the production strain *E. coli* BLASC, the stability of the genetic traits in the GMM was demonstrated using 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 alpha amylase. Three independent batches were analysed 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.

6 ADDITIONAL INFORMATION RELATED TO THE SAFETY OF AN ENZYME PROCESSING AID DERIVED FROM A GENETICALLY-MODIFIED MICROORGANISM

6.1 Information on the methods used in the genetic modification of the source microorganism

6.1.1 *CHARACTERISTICS OF THE VECTORS*

Vector systems used during the process of generating the recombinant *E. coli* BLASC are as described below:

1. **Generation of pTA**

Vector 1: pET21 as the backbone

2. **Generation of pTA-amyM**

Vector 2: pTA for cloning of *amyM*

Vector 1: pET21

pET21 is a bacterial expression vector with T7lac promoter, it adds N-terminal T7 epitope tag and C-terminal His tag. It contains the ampicillin resistance marker and multiple cloning sites (MCS) for cloning the gene of interest. For generating the vector pTA, the *ccdA* gene was cloned to the pET21 vector and the ampicillin resistance gene was deleted.

Vector 2: pTA (Cloning of synthesized *amyM*)

The vector pTA is a plasmid with *ccdA* gene, pBR322 and f (+) origin of replication. pTA was utilized for cloning of *amyM* gene to generate pTA-amyM for expression in *E.coli* SAML27.

6.1.2 *INTRODUCED DNA*

Marker gene: The *ccdB* and *ccdA* genes were amplified by PCR technique from the F plasmid of *Escherichia coli*. The Pmob promoter controlling the expression of the *ccdA* gene was amplified by PCR from the pBHR1 plasmid.

Synthetic DNA: The maltogenic alpha amylase gene (*amyM)* used to genetically modify the recipient *E.coli* SAML27 is a synthetic gene encoding the protein sequence of *Geobacillus stearothermophilus*. Codons were optimized to enhance protein expression in *E. coli*. Since *amyM* gene is a heterologous gene for *E. coli*, it is necessary to optimize it in order to maximize protein production. Only bases were changed at the DNA level, no amino acids are changed. The sequence encoding codon 2 to 33 (4 - 99 bases) of the gene were removed since this part encodes for signal peptide.

6.1.3 *DESCRIPTION RELATING TO THE GENETIC MODIFICATION*

The genetic modification for generation of recombinant production strain *E. coli* BLASC producing maltogenic alpha amylase enzyme consisted of three steps:

1. Generation of host strain *E.coli* SAML27 by integration of *ccdB* gene suitable for post-segregational killing system.
2. Generation of plasmid pTA-amyM containing the Maltogenic alpha amylase gene.
3. Transformation of plasmid pTA-amyM to *E. coli* SAML27 to generate recombinant production strain *E. coli* BLASC producing Maltogenic alpha amylase enzyme.

The strategy for generating recombinant *E. coli* BLASC producing maltogenic alpha amylase enzyme, involved the generation of post-segregational killing system and generation of recombinant plasmid containing the maltogenic alpha amylase gene.

Marker system: The *ccdB* and *ccdA* genes were amplified by PCR technique from the F plasmid of *Escherichia coli.* The Pmob promoter controlling the expression of the *ccdA* gene was amplified by PCR from the pBHR1 plasmid (Szpirer and Milinkovitch, 2005). The *ccdB* gene was integrated into the genome and the *ccdA* gene was cloned to the plasmid.

Recombinant plasmid: The synthetic *amyM* (2064 bp) gene was cloned to pTA (devoid of antibiotic marker) to generate pTA-amyM (6913 bp).

No functional antibiotic resistance genes were introduced into the host during genetic modification and hence the strain is free from any antibiotic gene. (Annex I Conf.)

6.1.4 INFORMATION RELATING TO THE PRODUCT PURIFICATION PROCESS

Absence of the production organism in the product:

Absence of production organism (*E. coli* BLASC) in the product was demonstrated as per EFSA Journal, 2011 (EFSA Journal 2011; 9(6):2193).

Three production batches of the maltogenic alpha amylase enzyme were analysed for the presence/absence of the production microorganism. The results indicated that the test samples did not show any colony for production strain on petri plates. The positive control, viz the enzyme sample (1 g) spiked with one cell showed growth indicating the technique is efficient to detect even the presence of 1 cell in the enzyme sample. The results indicated absence of recombinant production strain *E. coli* BLASC in all the three batches analysed.

Absence of transferable recombinant DNA sequences in the enzyme preparation:
Absence of transferable recombinant DNA sequences in the product was demonstrated as per EFSA Journal, 2011. Following parameters were considered during the method development:

1. Total DNA extraction procedure incorporates the cell lysis step to ensure that all DNA present in the sample is extracted.
2. Optimize a method for determining the quantification limit of the PCR assay.
3. Determining the detection limit of recombinant DNA in the sample by spiking the control DNA in different dilutions until DNA extinction before commencing the DNA extraction process.

Maltogenic alpha amylase produced by genetically modified microorganism was analysed for the presence of any transferable recombinant DNA sequence using PCR based detection method for gene fraction spanning 1kb of the PCR product. A DNA extraction method was developed considering the fact that enzyme products have higher protein contents, which can influence DNA recovery. In addition, 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 alpha amylase enzyme samples, DNA was extracted from enzyme preparation of maltogenic alpha amylase and also from enzyme preparation spiked with known amount of DNA (maltogenic alpha amylase enzyme preparation positive control). Further, to check the factors or inhibitors, which can cause PCR failure in extracted DNA from enzyme preparation, total DNA of known amount from the recombinant production strain *E. coli* BLASC spiked in extracted DNA from enzyme preparation was taken as positive control. PCR positive and negative controls were included to ensure functional PCR.

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

6.1.5 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 SAML27 bacteria for the expression of *amyM* gene and eventually the production of maltogenic alpha amylase.

The production strain *E. coli* BLASC contains the *ccdA* and *ccdB* genes to stabilize the plasmid. These two small genes were amplified by PCR technique from the F plasmid of *E. coli* (non-pathogen). The target of the *ccdB* gene is the bacterial DNA gyrase. This enzyme is not present in eukaryotic cells and consequently, *ccdB* has no action on these cells. *CcdA* encodes the ccdA antidote protein able to counteract the action of the ccdB protein. CcdA

protein is also able to repress the expression of the *ccdB* gene. Consequently, no expression of the *ccdB* gene is observed when ccdA protein is produced by the plasmid. CcdA does not present any toxicity for mammalian cells even when overproduced (Reschner et al., 2013).

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 alpha amylase involved cloning of the maltogenic alpha amylase gene (*amyM*) to the expression vector pTA which utilizes the *ccdA* and *ccdB* system to stabilize the plasmid. The vector is completely stable in the production host even without antibiotic resistance due to the stabilization system based on post-segregational killing system. The expression of maltogenic alpha amylase is episomal and the introduced DNA remains on the vector transformed to *E. coli* SAML27.

7 INFORMATION RELATED TO THE DIETARY EXPOSURE TO THE PROCESSING AID

7.1 A list of foods or food groups likely to contain the processing aid or its metabolites.

According to the food group classification system used in the standard 1.3.1- Food Additives Schedule 15(15-5), the maltogenic alpha amylase will be used in:

7. Breads and bakery products
14.2 Alcoholic beverages (including alcoholic beverages that have had the alcohol reduced or removed)
6.2 Flours, meals and starches

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

Maltogenic alpha amylase is used as a processing aid during starch processing, baking and brewing catalyses the hydrolysis (1→4)-alpha-D-glucosidic linkages in polysaccharides.

Baking:

During baking, the maltogenic alpha amylase food enzyme 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 alpha 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 J.E.C. et.al. 2002).

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

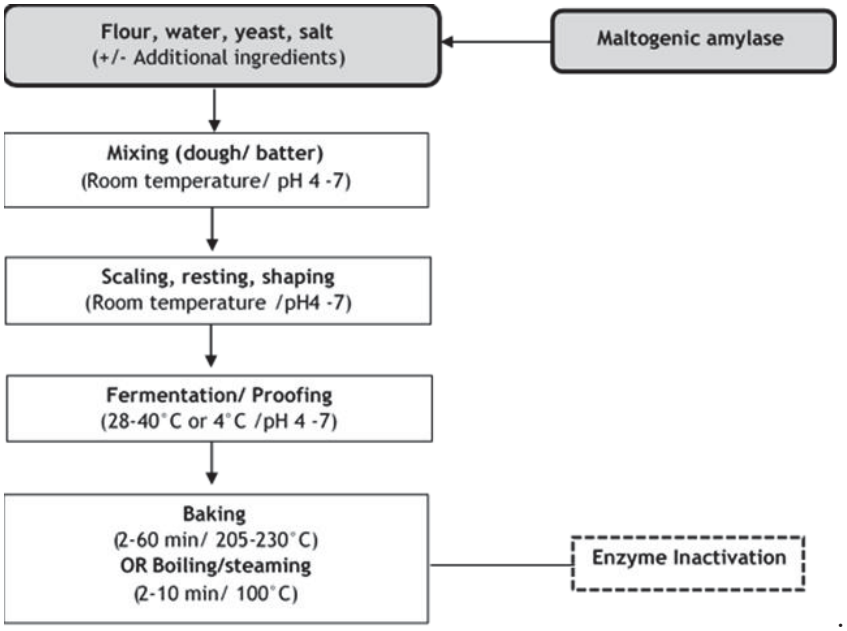


Fig. 7: Flow-chart - Use and fate of the maltogenic alpha amylase in baking

Starch processing for 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 syrup.

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

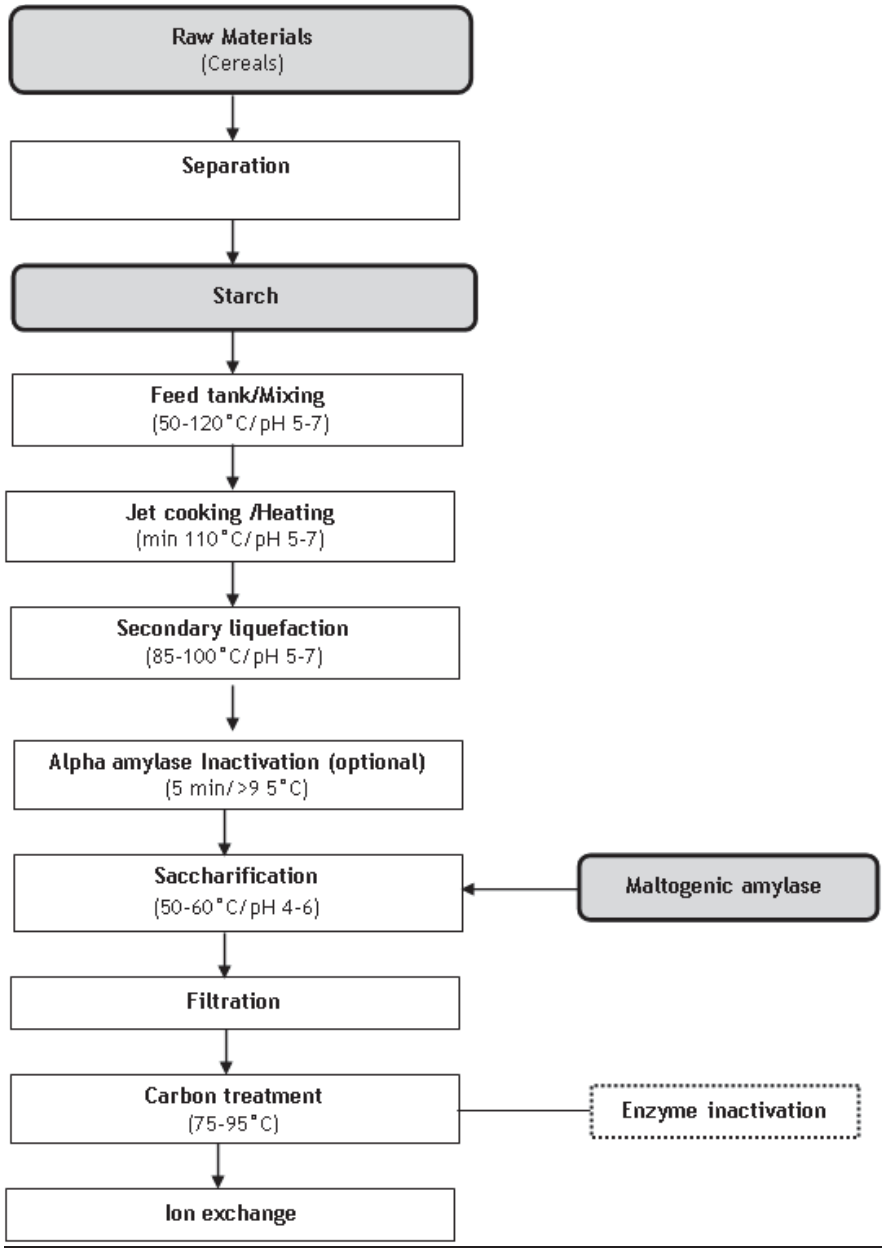


Fig 8: Flow-chart - Use and fate of the maltogenic alpha amylase in starch processing

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 from use of maltogenic alpha amylase are improved yields due to 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 alpha amylase in brewing is as shown below.

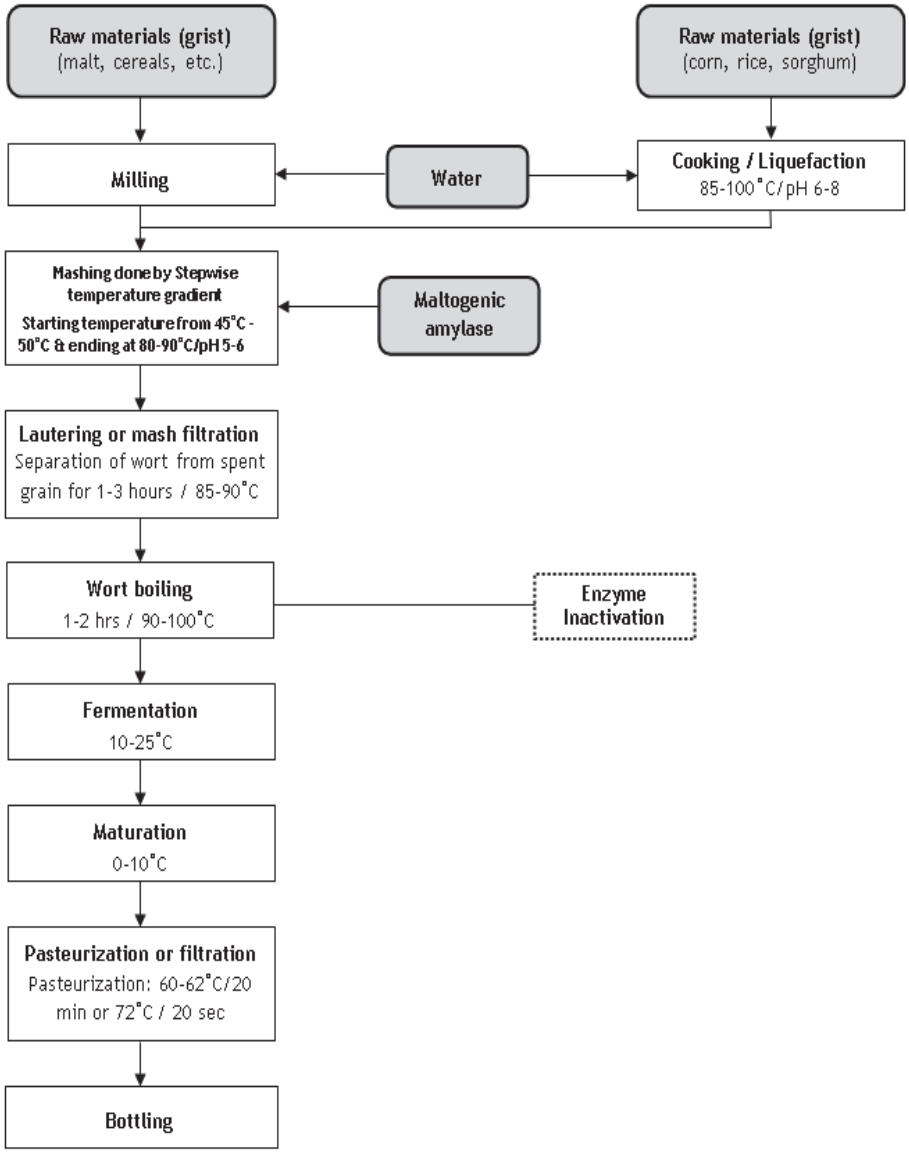


Fig 9: Flow-chart - Use and fate of the maltogenic alpha amylase in brewing

7.2 The levels of residues of the processing aid or its metabolites for each food or food group

USE LEVELS

Recommended use levels of the food enzyme maltogenic alpha amylase

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.

ACTIVE AND INACTIVE ENZYME RESIDUES IN THE FINAL FOOD

Maltogenic alpha amylase performs its technological function during food processing, it does not perform any technological function in the final food. The reasons why the maltogenic alpha 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 alpha amylase in various processes, are explained in more details below:

Baking:

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

Starch processing for production of maltose/glucose syrup:

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

Brewing:

In brewing processes, the maltogenic alpha amylase is added during mashing, before lautering or mash filtration. At the end of the mashing, maltogenic alpha amylase is inactivated by

increasing temperature of mash; further, it is also denatured in the consecutive lautering or mash filtration and wort boiling steps.

ESTIMATES OF HUMAN CONSUMPTION

The maltogenic alpha 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 this wide variety of applications, the most appropriate way to estimate the 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 1) the level of consumption of foods and of non-milk beverages, 2) the concentration of the additive in foods and in non-milk beverages and 3) the proportion of foods and of non-milk beverages that may contain it.

Table 5: Maximal level of food enzyme maltogenic alpha 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

To calculate the TMDI, the maximum use levels in individual processes have been 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 levels of consumption of foods and beverages considered are maximum physiological levels of consumption—i.e. the daily consumption of 0.1 litre of non-milk beverages per kilogram of body weight and the daily consumption of 100 kcal/kg body weight from foods (equivalent to 0.05 kg/kg body weight based on an estimated energy density of 2 kcal/g) (Hansen, 1979). The levels contained in foods and beverages are assumed to be the highest maximum levels of the additive reported in any category for foods and for beverages, respectively. The proportions of solid foods and beverages that may contain the substance are set arbitrarily. In the case of food additives, a default proportion that is often used for European assessments is 12.5% for solid foods and 25% for beverages (WHO EHC Chapter 6, 2009).

The overall theoretical maximum daily exposure to an additive is calculated by summing the potential exposure from beverages and from foods, as shown below:

Overall theoretical maximum daily Exposure = [maximum level of the additive in beverages (mg/l) × 0.1 (litre/kg body weight) × percentage of beverages that may contain the substance]

+ [maximum level of the chemical in solid foods (mg/kg) × 0.05 (kg/kg body weight) × percentage of solid foods that may contain the substance]

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)
33.892 x 0.05 x 0.125 = **0.2118**	33.892 x 0.1 x 0.25 = **0.8473**	**1.0591**

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 producer of the above-mentioned foodstuffs and beverages use the specific enzyme maltogenic alpha amylase.
- It is assumed that producer apply the highest use level per application;
- For the calculation of the TMDI's in food as well as in beverage, 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 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 NOAEL dose for maltogenic alpha amylase from *E coli* BLASC, concluded from the 90-day toxicity study is 837.9 mg TOS/kg body weight (Considering 83.79% TOS), corresponds to 144,255 MANU/ /kg body weight. Acceptable Daily Intake (ADI) concluded from the NOAEL dose, is 586.53 mg TOS/person/day or 100978.5 MAN U/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 74.14 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 CALCULATION

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL by the estimated human consumption value. As was shown above, the estimated human consumption value of the food enzyme is **1.0593 mg TOS/kg body weight/day.**

Margin of Safety (MoS) = 837.9/1.0591 = 791.1434 i.e.791

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. Therefore, the actual MoS in practice will be some magnitudes higher. Consequently, there are no safety reasons for laying down maximum levels of use.

7.3 For food or food groups not currently listed in the most recent Australian or New Zealand national nutrition surveys (NNSs), information on the likely of consumption

Not relevant.

7.4 The percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid

It is assumed that all raw materials containing starch are processed using the maltogenic alpha amylase, object of this dossier as a processing aid at the highest recommended dosage.

7.5 Information relating to the levels of residues in foods in other countries

As described in section 7.2, a “worst case” calculation is made assuming that all organic matter originating from the enzyme is retained in the processed food product and an exaggerated human intake is estimated using on the Budget method. In aforementioned applications, the amount of maltogenic alpha amylase and TOS present in the final food is negligible.

Applications and levels of use of the maltogenic alpha amylase preparation in other countries is the same as presented in section 7.2. The European Food Safety Agency has evaluated and expressed no safety concern for the same applications and use levels of maltogenic alpha amylase.

7.6 For foods where consumption has changed in recent years, information on likely current food consumption

No significant changes in recent years are observed.

8 ANNEXES

Annex	Description	Reference	Pages
Annex A1	Method of Analyses	-	1-20
Annex A2	Analytical method for measuring the activity of maltogenic alpha amylase (EC 3.2.1.133)	-	1-2
Annex B	Heavy Metals Analysis – TUV Nord Group	41400019, 71400440, 71400441	1-3
Annex C	Mycotoxin Analysis – TNO Triskelion BV	20121-0815,16,17	1-3
Annex D	Allergen declaration for maltogenic amylase	-	1
Annex E Conf.	Deposition certificate of production strain BLASC	-	1
Annex F Conf.	Identification information of Strain BLASC	AML 081A	1-5
Annex G1	Acute oral toxicity of maltogenic amylase in rats	Intox-14164	1-29
Annex G2	Repeated dose 90-day oral Toxicity study with maltogenic amylase by daily gavage in the rat followed by a 4 week recovery period	Intox-14165	1-175
Annex G3	Bacterial reverse mutation test of maltogenic amylase	Intox-14166	1-50
Annex G4	*In vitro* mammalian chromosome aberration test of maltogenic amylase in human lymphocytes	Intox-14167	1-54
Annex H	EFSA scientific opinion - Safety evaluation of the food enzyme maltogenic amylase from genetically modified *Escherichia coli* (strain BLASC)	_	1-16
Annex I Conf.	Description of the genetic modification for generation of the maltogenic alpha amylase production strain *Escherichia coli* BLASC	_	1-20
Annex J	Confidential commercial Information	_	2
Annex K	Statutory Declaration	_	1
Annex L	Allergenicity Assessment – Maltogenic amylase from GMM E. Coli (strain BLASC)	_	1-10
Annex M1	Action of maltogenic amylase from *E. coli* BLASC on amylopectin and reaction products	MAA/AET/FAL/001	1-9
Annex M2	Effect of maltogenic amylase from E. coli BLASC on loaf bread crumb softness, slice resilience, loaf volume, crumb texture and shelf life	MAA/AET/FAL/003	1-13
Annex M3	Action of maltogenic amylase from *E. coli* BLASC for corn starch modification to produce maltose syrup	MAA/AET/FAL/004	1-10

Annex M4	Action of maltogenic amylase from E. coli BLASC in brewing process using combination of Barley malt and millet.	MAA/AET/FAL/005	1-13
Annex M5	Action of maltogenic amylase from *E. coli* BLASC on the hydrolysis of starch from various grains	MAA/AET/FAL/002	1-9
Annex N	Three batches COAs of Maltogenic alpha amylase	_	1-3

9 BIBLIOGRAPHY-REGULATIONS / REPORTS BY IMPORTANT AGENCIES (BBR)

1. Amraini SZ, Ariyani LP, Hermansyah H, *et al.* 2017. Production and characterization of cellulase from *E. coli* EgRK2 recombinant based oil palm empty fruit bunch. Biotechnol Bioproc E 22: 287–295
2. Arrêté du 19 Octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la fabrication de certaines denrées alimentaires; Legifrance (2020)
3. Australia New Zealand Food Standards Code - Schedule -15 Substances that may be used as food additives (December, 2019)
4. Australia New Zealand Food Standards Code - Standard 1.3.3 - Schedule -18 Processing Aids (September, 2020)
5. Barley Malt Beer, Agribusiness Handbook; FAO Investment Centre Division (2009) http://www.fao.org/3/a-i1003e.pdf
6. 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
7. 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
8. Brumm P. J. & Teague W. M; A reduced stability *Bacillus stearothermophilus* α-amylase for food applications. Biotechnology Letters volume 10(7), 445–450 (July 1988).
9. 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
10. 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
11. Chinese Standards for Food Additives - GB2760-2015: National Food Safety Standard for Food Additive Use, implemented on May 24, 2015
12. 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.
13. 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
14. 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
15. 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.

16. 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.
17. EFSA (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
18. 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
19. 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
20. 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
21. EFSA Journal (2019), Safety evaluation of the food enzyme maltogenic amylase from genetically modified *Escherichia coli* (strain BLASC), EFSA Journal 2019;17(5):5769
22. 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
23. ETA position on 'Food Allergen Labeling of Microbially Derived Enzymes' Under FALCPA as it Applies to Fermentation Media Raw Materials'(2005)
24. FALCPA, Food Allergen Labeling And Consumer Protection Act of 2004
25. FAO/JECFA (2006a). Joint FAO/WHO Expert Committee on Food Additives Monographs: Combined compendium of food additive specifications, Volume 4, pp112
26. 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)
27. FARRP / AllergenOnline | http://www.allergenonline.org/databasefasta.shtml
28. 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
29. Food Standards Australia New Zealand Application Handbook (1 July 2019)
30. 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
31. 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
32. Gopal GJ, Kumar A. Strategies for the production of recombinant protein in *Escherichia coli*. Protein J. 2013;32(6):419–425
33. 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

34. Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B (2003) Microbial a-amylases:a biotechnological perspective. Process Biochemistry, 38, 1599-1616
35. 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
36. Hansen SC; Acceptable daily intake of food additives and ceiling on levels of use.; (1966); Food Cosmet Toxicol; 4; 427–432
37. Hansen, S.C. (1979). Conditions for use of food additives based on a budget method for an acceptable daily 16 intake. Journal of Food Protection, 42, 429-434.
38. 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)
39. 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.
40. JECFA monograph (1999), Maltogenic amylase from *Bacillus stearothermophilus* expressed in *Bacillus subtilis*
41. 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
42. Jia B, Jeon CO. High-throughput recombinant protein expression in *Escherichia coli*: current status and future perspectives. Open Biol. 2016;6:8
43. Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology; 22 – 25 January 2001; Topic 5: Stability of Known Allergens (Digestive and Heat Stability) | http://www.fao.org/ag/agn/food/pdf/bi07al.pdf
44. Kamionka M. 2011. Engineering of therapeutic proteins production in *Escherichia coli*. Curr Pharm Biotechnol. 12(2):268–74
45. 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
46. Korea Food Additives Code (2004). Maltogenic amylase Assay.
47. 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
48. 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.
49. 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.

50. 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.
51. 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
52. 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.
53. 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.
54. OECD Principles on Good Lab Practice (1998) Series on Principles of Good Laboratory Practice and Compliance Monitoring; 1-41
55. 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
56. 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
57. OECD Test No. 471: Bacterial Reverse Mutation Test (1997) OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing; 1-11
58. OECD Test No. 473: In Vitro Mammalian Chromosomal Aberration Test (1997) OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing; 1-10
59. Pence JW (1953) Panary Fermentation. Current Status of Problems. Journal of Agricultural and Food Chemistry, 1 (2), 157-161.
60. 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.
61. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology;22 – 25 January 2001; 1-29
62. Reschner A., Scohy S., Vandermeulen G., Daukandt M., Jacques C., Michel B., Nauwynck H., Xhonneux F.,Préat V., Vanderplasschen A., Szpirer C. (2013),Use of Staby® technology for development and production of DNA vaccines free of antibiotic resistance gene. Hum Vaccin Immunother. 2013 Oct 1; 9(10): 2203–2210
63. 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.
64. Rosano GL, Ceccarelli EA. Recombinant protein expression in Escherichia coli: advances and challenges. Front Microbiol. 2014;5:172.
65. Slominska L. Enzymatic Modification of Low Conversion Starch Products. Starch; Volume41, Issue5, 1989 ;Pages 180-183
66. 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

67. Sweetness from Starch; FAO Corporate Document Repository (1996) http://www.fao.org/3/v9805e/V9805E00.htm
68. 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.
69. The Fresh Loaf Baker's Handbook http://www.thefreshloaf.com/handbook
70. 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.
71. USFDA GRN 746 GRAS Notification for Maltogenic amylase from *Geobacillus stearothermophilus* produced in *Bacillus subtilis*. AB Enzymes, Germany (2018).
72. USFDA GRN 751 GRAS Notification for Maltogenic alpha-amylase *from Bacillus stearothermophilus* produced in *Bacillus subtilis*. Novozymes North America (2018).
73. USFDA GRN 842 GRAS Notification for A Maltogenic a-amylase from *Geobacillus stearothermophilus* produced by *Saccharomyces cerevisiae*. Mascoma LLC (2019).
74. USP (2008). Unites States Pharmacopeia, the National Formulary USP31, NF26. Biological Tests and Assays: Antibiotics–Microbial Assay, pp91-96. Rockville, MD, USA.
75. 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
76. 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.
77. WHO EHC Chapter 6, Dietary Exposure Assessment of Chemical in Foods (2009).