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January 7, 2009

Dr. Laura Tarantino, Director
Office of Food Additive Safety, HFS-200
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Dear Dr. Tarantino:

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. 170.36, Genencor, a Danisco Division, ("Danisco") has determined that its amylase enzyme preparation produced by *Bacillus licheniformis* expressing a variant of the gene encoding maltotetraohydrolase (PS4wt), an amylase, from *Pseudomonas saccharophila* is a Generally Recognized as Safe ("GRAS") substance for the intended food application and is, therefore, exempt from the requirement for premarket approval. We are hereby submitting, in triplicate, a Generally Recognized As Safe ("GRAS") Notification, in accordance with proposed 21 C.F.R. § 170.36, informing FDA of the view of Danisco that the amylase enzyme preparation is GRAS, through scientific procedures, for use as an enzyme to delay the staling process in bread and other baked goods and thereby extend the period for which the products have an acceptable eating quality. This is a benefit to the bread producers and to consumers as bread will keep fresh for a longer time and disposal of stale bread or baked goods is minimized. The ability of this amylase to form maltotetraose can also be exploited in the starch processing industry. In these applications the enzyme is considered a processing aid.

If you have questions or require additional information, please contact me at 650-846-7557 or fax at 650-845-6505.

Sincerely,

Alice J. Caddow
Vice President of Regulatory
And Environmental Affairs

Enclosures (3 binders)

G4-amylase Enzyme Preparation from *Bacillus licheniformis* Expressing a Modified Maltotetraohydrolase (G4-amylase) Gene from *Pseudomonas saccharophila* Is Generally Recognized As Safe For Use in Food Processing

Notification Submitted by Genencor, a Danisco Division

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1. GENERAL INTRODUCTION

The G4-amylase enzyme preparation under consideration is produced by submerged fermentation of *Bacillus licheniformis* carrying the gene encoding a protein engineered variant of the maltotetraohydrolase enzyme from *Pseudomonas saccharophila*. The *mta* gene encoding the native maltotetraohydrolase PS4wt from *P. saccharophila* was cloned by PCR based on the mta sequence[1]. To avoid heterogeneity of the G4-amylase upon limited proteolysis, the starch-binding domain was removed by introducing a stop codon after residue G429.

Subsequently, this G4-amylase has been developed by successive cycles of random, site specific mutagenesis and site scanning mutagenesis combined with screening for thermostability and exo-specificity. This G4-amylase contains sixteen amino acid changes compared to the sequence of the catalytic core of PS4wt.

By introducing these mutations, the temperature stability and baking performance of the enzyme is increased, making this G4-amylase far better suited for anti-staling applications than the wild-type maltotetraohydrolase enzyme from *P. saccharophila*.

This G4-amylase will be used to delay the staling process in bread and other baked goods and thereby extend the period for which the products have an acceptable eating quality. This is a benefit to the bread producers and to consumers, as bread will keep fresh for a longer time and disposal of stale bread and baked goods is minimized. The ability of this G4-amylase to form maltotetraose can also be exploited in the starch processing industry. In these applications the enzyme is considered a processing aid.

The systematic name of the principle enzyme activity is 4-α-D-Glucan maltotetraohydrolase. Other names used are glucan 1,4-α-maltotetraohydrolase, exo-maltotetraohydrolase, maltotetraohydrolase, maltotetraose-forming amylase, and G4-amylase.

The enzyme catalyses hydrolysis of 1,4-α-D-glucosidic linkages in amylaceous polysaccharides to remove successive maltotetraose residues from the non-reducing chain ends.

The EC number of the enzyme is 3.2.1.60, and the CAS number is 37288-44-1.

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. 170.36, Danisco has determined that its G4-amylase enzyme preparation from a modified strain of *B licheniformis* expressing a protein engineered maltotetraohydrolase enzyme from *P. saccharophila* is a Generally Recognized As Safe ("GRAS") substance for the intended food application and is, therefore, exempt from the requirement for premarket approval. The information provided in the following sections is the basis of our determination of GRAS status of this G4-amylase enzyme preparation produced by a *B. licheniformis* host, which has been modified to express a gene encoding a protein engineered maltotetraohydrolase enzyme from *P. saccharophila*.

Our safety evaluation in Section 7 includes an evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure to the preparation.

The safety of the production organism must be the prime consideration in assessing the safety of an enzyme preparation intended for food use.[2,3] The safety of the production organism for this G4-amylase, *B. licheniformis,* is discussed in Sections 2 and 7 Another essential aspect of the safety evaluation of enzymes derived from genetically modified microorganisms is the identification and characterization of the inserted genetic material.[4-9] The genetic modifications used to construct this production organism are well defined and are described in Section 2. The safety evaluation described in Section 7 shows no evidence to indicate that any of the cloned DNA sequences and incorporated DNA code for or express a harmful toxic substance.

GENENCOR

1.1 Name and Address of Notifier

Genencor, A Danisco Division
925 Page Mill Road
Palo Alto, CA 94304

1.2 Common or Usual Name of Substance

G4-amylase enzyme preparation from *B. licheniformis* expressing the gene encoding a protein engineered variant of the maltotetraohydrolase enzyme from *Pseudomonas saccharophila.*

1.3 Applicable Conditions of Use

This G4-amylase will be used to delay the staling process in bread and other baked goods and thereby extend the period for which the products have an acceptable eating quality. This is a benefit to the bread producers and to consumers as bread will keep fresh for a longer time and disposal of stale bread and baked good is minimized. The ability of this G4-amylase to form maltotetraose can also be exploited in the starch processing industry. In these applications the enzyme is considered a processing aid.

1.4 Basis for GRAS Determination

This GRAS determination is based upon scientific procedures.

1.5 Availability of Information for FDA Review

A notification package providing a summary of the information which supports this GRAS determination is enclosed with this notice. The package includes a safety evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure. Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying upon request.

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The production organism of the G4-amylase enzyme preparation is *B. licheniformis* strain GICC03279. It is derived by recombinant DNA methods from a strain of *B. licheniformis* modified to express a modified G4-amylase gene DNA (see Section 2.2). *Bacillus licheniformis* is considered a Class 1 Containment Agent under the NIH Guidelines for Research Involving Recombinant DNA Molecules and by the Ministerie van de Vlaamse Gemeenschap in Flanders, Belgium, and is also considered as GILSP worldwide. It also meets the criteria for a safe

production microorganism as described by Pariza and Johnson[2] and several expert groups.[3-9] It contains the modified G4-amylase gene under the regulation of a native *B. licheniformis* promoter and terminator along with a selectable marker, the native *B. licheniformis cat* gene.

The inserted DNA was integrated into the host on a in a vector derived from *Bacillus* plasmids pUB110 and pE194. The introduced DNA integrated into the Bacillus chromosome at the *cat* locus by Campbell type recombination After integration all vector sequences of the plasmid were deleted by recombination between direct repeated *cat* sequences

2.2 Host Microorganism

The host organism is *B. licheniformis* BRA7. *B. licheniformis* BRA7 was modified through deletion of several enzyme activities (proteases, amylase), a sporulation gene and the native chloramphenicol resistance genes to make it suitable for expression of G4-amylase. This strain lineage has been used by Genencor as a host for the commercial production of a number of α-amylases for the starch liquefaction business since 1989, as well as for production of protease, pullulanase, xylanase, and glycerophospholipid cholesterol acyltransferase. The strain has a sporulation frequency of less than 10^{-8} as determined by comparing the titer of the colony forming units (CFU) in the culture before and after heating at 85° C for 10 minutes. The strain, which has a history of safe large-scale fermentation, has been typed as *B. licheniformis* based on 16S rDNA gene sequencing and ribotyping.

2.3 G4-amylase Expression Vector

The vector used in this construction contains the following features. a temperature sensitive origin of replication (*ori* pE194, for replication in *Bacillus*), *ori* pBR322 (for amplification in *Escherichia coli*), the pUB110 neomycin resistance gene (*neo*) for initial selection, and the native *B. licheniformis* chloramphenicol resistance gene (*cat*) for selection, chromosomal integration and cassette amplification. The *cat* gene is present on a larger native *B. licheniformis* chromosomal fragment surrounding the coding sequence with upstream and downstream sequences. Part of the upstream *cat* sequence (called 5' repeat) is present twice on the plasmid to allow amplification of the expression cassette on the chromosome. The expression cassette (containing a native promoter and signal sequence and the G4-amylase gene) is located between these repeats.

The genetic construction was evaluated at every step to assess the incorporation of the desired functional genetic information and the final construct was verified by Southern blot analysis to confirm that only the intended genetic modifications to the *B. licheniformis* strain had been made.

2.4 Stability of the Introduced Genetic Sequences

The production strain proved to be 100% stable after at least 60 generations of fermentation, judged by chloramphenicol resistance and G4-amylase production

2.5 Antibiotic Resistance Gene

The construction of the production organism utilized the native chloramphenicol acetyltransferase (*cat*) gene from *B. licheniformis* conferring chloramphenicol resistance; no new antibiotic resistance was introduced.

2.6 Absence of Production Microorganism in Product

The absence of the production microorganism is an established specification for the commercial product at a detection limit of 1CFU/g. The production organism does not end up in food and therefore, the first step in the safety assessment as described by the IFBC[4] is satisfactorily addressed.

3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1 Enzyme Identity

Classification	Glycoside hydrolase
IUB Nomenclature	4-α-D-Glucan maltotetraohydrolase
IUB Number:	3.2.1.60
CAS Number·	37288-44-1
Reaction catalyzed	hydrolysis of 1,4-α-D-glucosidic linkages in amylaceous polysaccharides to remove successive maltotetraose residues from the non-reducing chain ends.

3.2 Amino Acid Sequence

The G4-amylase in question is a variant of the wild type maltotetraohydrolase (PS4wt) from *P. saccharophila* strain IAM 1504 (obtained from the Institute of Applied Microbiology (IAM) Culture Collection, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Yayoi, Bunkyo-Ku, Tokyo 113-0032, Japan). Compared to the wild type enzyme PS4wt encoded by the gene *mta* of *P. saccharophila* strain IAM 1504[1], the starch-binding domain has been removed and 16 out of the remaining 429 amino acids of the catalytic core have been changed in order to improve thermostability and baking performance of the enzyme.

The amino acid sequence of this G4-amylase is shown in Appendix 1.

3.3 G4-amylase Donor Safety

Pseudomonas saccharophila and *Pseudomonas stutzeri* are gram negative, rod shaped bacteria mobile by means of a single polar flagellum. They are obligate aerobes, except in media with nitrate. They do not require organic growth factors and are found in soil and water[10].

Even though it is only the gene encoding the maltotetraose forming amylase which is transferred from the donor organism to the host organism, the safety of the donor organism was assessed, as *P. stutzeri* and *P. saccharophila* do not have a long history of safe use in the food industry. The safety was assessed based on search in the scientific literature.

The databases Medline, Chemtox and Chemical Abstract were searched. As the donor organism was identified as *P. saccharophila* by IAM, but as *P. stutzeri* based on 16s rDNA analysis, both the words "Pseudomonas" and "stutzeri or saccharophila" were used. These were combined with "pathogen or patogen or allergen or allergic or allergy or toxin or toxic or toxicology" for the period 1980 to 26 June 2006.

Reisler and Blumberg[11] reviewed the literature in the English language in the period January 1966 through August 1998 and reported 29 cases of *P. stutzeri* infections in humans. Among these cases were 2 deaths. Both patients were immunocompromised and in one case the death may not be directly related to the initial infection. They reported one case of community-acquired vertebral osteomyelitis caused by *P. stutzeri* and described it to be the first recorded case of a community-acquired, atraumatic infection in a previously healthy, non-immunocompromised person. The patient was successfully treated with antibiotics.

The database search revealed 3 other published papers on human infection cases with *P. stutzeri* (Campos-Herrero *et al*[12]; Potvliege *et al*[13] Puzenat *et al*[14]. All 3 patients were cured for their *P. stutzeri* infection by antibiotics. Two of the cases were included in the Reisler and Blumberg review, while one was reported more recently.

Lin *et al*[15] reported the isolation of *P. stutzeri* from the respiratory tract or bone marrow of dead chickens amongst 12 other Pseudomonas species and two other gram-negative hemolytic bacteria. Of the 15 species, 7 caused more than 50% mortality in chickens in an experimental set-up, while *P. stutzeri* resulted in less than 30% mortality. All strains including *P. stutzeri* were susceptible to some antibiotics and resistant to others.

Cortese *et al*[16] reported that *P. stutzeri* strain KC contains the MoeZ gene, which is essential for the synthesis of pyridine-2,6-bis(thiocarboxylic acid) (pbtc), a potential pathogenicity factor. However, other *P. stutzeri* strains and other Pseudomonas species tested lacked the pbtc synthesis genes. Cortese *et al*[17] showed that *P. stutzeri* strain KC and *P. putida* DSM301 are able to produce pbtc, which is involved in the overall level of iron uptake, but they do not present any correlation to potential pathogenicity. Sebat *et al*[18] also describes *P. stutzeri* strain KC as a pbtc-forming organism and the strain demonstrated antagonism towards *E. coli*.

Martins *et al*[19] reports that *P. stutzeri* in some papers has been reported as capable of producing paralytic shellfish toxins (PSTs), but that results of autonomous (not related to presence of dinoflagellates) production is controversial From their results in this paper the researchers do not conclude that autonomous production of PSTs by Pseudomonas can be excluded, but they show that utmost care must be taken in interpreting results and that previous findings have been false positives. Baker *et al*[29] reports that they can not rule out the possibility that *P. stutzeri* strain SF/PS produces saxitoxins; however, their data clearly demonstrate that the strain accumulates at

least five different fluorescent compounds that could be easily mistaken for the saxitoxin derivative GTX_4.

4. MANUFACTURING PROCESS

This section describes the manufacturing process for this G4-amylase which follows standard industry practice [20-22] For a diagram of the manufacturing process, see Appendix 2 The quality management system used in the manufacturing process complies with the requirements of ISO 9001. The enzyme preparation is also manufactured in accordance with FDA's current Good Manufacturing Practices ("cGMP") as set forth in 21 C.F.R Part 110.

4.1 Raw Materials

The raw materials used in the fermentation and recovery process for this G4-amylase concentrate are standard ingredients used in the enzyme industry [20-22] All the raw materials conform to the specifications of the Food Chemicals Codex, 6th edition, 2008 ("FCC"),[23] except for those raw materials which do not appear in the FCC For those not appearing in the FCC, internal requirements have been made in line with FCC requirements and acceptability of use for food enzyme production. Danisco uses a supplier quality program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and are verified upon receipt.

The antifoam and flocculants used in the fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 24, 1998. The maximum use level of these antifoam in the production process is $\leq$0.15% and of the flocculants <1 5%.

4.2 Fermentation Process

The G4-amylase is manufactured by submerged straight-batch or fed-batch pure culture fermentation of the genetically modified strain of *B licheniformis* described in Section 2 All equipment is carefully designed, constructed, operated, cleaned and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are conducted periodically to ensure absence of foreign microorganisms and confirm production strain identity.

4.2.1 Production organism

A new lyophilized stock culture vial of the *B. licheniformis* production organism described in Section 2 is used to initiate the production of each batch. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

4.2.2 Criteria for the rejection of fermentation batches

Growth characteristics during fermentation are observed microscopically. Samples are taken from each fermentation stage (inoculum, seed and main fermenter) before inoculation, at regular intervals during growth and before harvest or transfer. These samples are tested for microbiological contamination by plating on a nutrient medium.

A fermentation batch is declared as 'contaminated' if colony forming units (CFU) of bacteria or fungi other than the production strain are present at levels $>10^3$CFU/ml.

If a fermentation batch is determined to be contaminated, it will be rejected if deemed appropriate. If the contamination is minor and determined to be from common non-pathogenic environmental microbes, the fermentation may be processed.

4.3 Recovery Process

The recovery process is a multi-step operation which starts immediately after the fermentation process and consists of both concentration and formulation processes.

4.3.1 Concentration process

The enzyme is recovered from the culture broth by the following series of operations:

1. Primary separation –centrifugation or filtration
2. Polish filtration – for removal of residual production strain organisms and as a general precaution against microbial degradation
3. Concentration – ultrafiltration
4. Precipitation
5. Centrifugation
6. Formulation
7. Polish and sterile filtration
8. Concentration.

4.3 2 Formulation and standardization process

The ultrafiltered concentrate is stabilized with sodium benzoate. The preserved ultrafiltered concentrate is then dried and agglomerated using any one of the common drying methods, such as spray drying, fluid bed agglomeration or fluid bed spray drier.

4 3 3 Quality control of finished product

The final G4-amylase liquid concentrate from *B. licheniformis* is analyzed in accordance with the general specifications for enzyme preparations used in food processing as

established by the Joint FAO/WHO Expert Committee on Food Additives ("JEFCA")[24] in 2006 and the FCC[23]. These specifications are set forth in Section 5.

The dried product is tested for enzyme activity, Total Viable Count and Dry Matter. The other assays conducted on the liquid concentrate are not repeated for the dried product. The dried product also is in compliance with the general specifications for enzyme preparations used in food processing as established by JEFCA[24] in 2006 and the FCC[23].

5. COMPOSITION AND SPECIFICATIONS

5.1 Quantitative Composition

The specifications for the dried product are:

Activity for powder for tablets	>1600 BMK/g
Activity for powder for powder blends	1000 - 1500 BMK/g
Total Viable Count	<50,000/g
Dry Matter	>91.0%

Dry Matter is measured by determining the loss on drying after 4 hours at 105°C and expressing as percentage change in weight.

5.2 Specifications

The G4-amylase preparation conforms to the general and additional requirements for enzyme preparations as described in the FCC[23]. In addition, the G4-amylase preparation also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by JEFCA[24].

The following specifications have been established for the G4-amylase:

(See next page)

Property	Method Number	Reference Method	Specification
ENZYME ACTIVITIES			
Amylase, betamyl units	Danisco/Genencor Method	150,000 BMU/g	Amylase, betamyl units
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	800V	ISO 4833 - "Microbiology - General guidance for the enumeration of micro-organisms - colony count technique at 30°C" and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Not more than 50,000 CFU/g
Total Coliforms	810V	ISO 4832 - "General guidance for the enumeration of coliforms - colony count technique" and the FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Not more than 30 CFU/g
E. coli	819V	ISO 7251 - Microbiology - "General Guidance for Enumeration of Presumptive *Escherichia coli* - Most Probable Number Technique" and FDA Bacteriological Analytical Manual, 8th Edition, AOAC International	Negative/25g
Salmonella	832V	Nordic Committee on Food Analysis; Salmonella Bacteria; Detection in Foods. No 71; 4th Edition; 1991 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Negative/25g
Production strain	892V	Genencor Method	Negative by test

Property	Method Number	Reference Method	Specification
Antibacterial Activity	899V	FAO Food and Nutrition Paper: 25th Session of the Joint FAO/WHO Expert Committee on Food Additives; Geneva 1981; p317-318, Appendix A	Negative by test
OTHER ASSAYS			
Arsenic	603W-AS	FAO Food and Nutrition Paper No. 5, GUIDE TO SPECIFICA-TION, General notices, General analytical techniques, Identification tests, Test solutions, and other reference materials, 1983, 2nd revision	Less than 3 mg/kg
Cadmium	603W-CD	Same as Arsenic	Less than 0.5 mg/kg
Mercury	603W-HG	Same as Arsenic	Less than 0.5 mg/kg
Lead	603W-PB	Same as Arsenic	Less than 5 mg/kg
Mycotoxins	604W	Patterson & Roberts, Assoc. of Anal. Chem. (vol. 62, no. 6, 1979)	Negative by test

Lead, arsenic, cadmium, mercury, antibacterial activity, and mycotoxins are analyzed at regular intervals. Activity and microbial specifications are analyzed on every production batch of enzyme.

The lead, Coliforms and Salmonella specifications meet FCC and JEFCA requirements. The *E. coli*, antibacterial activity and mycotoxin specifications meet JEFCA requirements and are not included in FCC (although FCC mentions mycotoxins, but has not established tolerances.) The production microorganism specification is a Danisco specification and is not mentioned in FCC or JEFCA.

The G4-amylase assay is colorimetric and monitors the rate of degradation of p-nitrophenyl maltoheptoside. Alpha-glucosidase and a glucoamylase are used as coupling enzymes. This substrate has the non-reducing terminal sugar chemically blocked, thus preventing any interaction with glucoamylase. The rate of p-nitrophenyl release is proportional to amylase activity and is monitored at 410nm. Note that this procedure involves a stop reaction at alkaline

pH. The alkaline pH is necessary to stop the reaction and to provide optimal color development for the endpoint reaction.

6. APPLICATION

6.1 Mode of Action

There are two main applications of amylases in baking. The first is flour standardization, *i.e.* optimization of α-amylase activity in the flour, and the second is anti-staling

Fungal α-amylase (EC 3.2.1.1) from *Aspergillus oryzae* is added to flour to optimise α-amylase activity. Use of fungal α-amylase in bread baking was approved in 1955 in the US and fungal α-amylase has since become the most widely used enzyme in baking world wide. Danisco started the marketing of fungal enzymes containing fungal α-amylase and xylanase activities to the milling and baking industry back in the 1980s.

The second important objective for use of amylases in baking is anti-staling, i.e., extending the freshness of baked goods. Staling is a highly complex phenomenon with firming being the most well-known and important symptom. Anti-staling amylases have to modify starch during the baking process to provide significant anti-staling effects. As intact starch granules are hardly attacked anti-staling amylases have to be sufficiently heat-stable to be active during the baking step after initial gelatinization of starch granules.

Endo-amylases (EC 3.2.1.1) are used for anti-staling and work by degrading amylose so that a weaker amylose network is formed during starch retrogradation after baking. Exo-amylases such as maltogenic amylase (EC 3.2.1.133) cloned from *Bacillus stearothermophilus* and now maltotetraohydrolase (EC 3.2.1.60) cloned from *P. saccharophila* offer clear improvements for anti-staling applications. By shortening the amylopectin side chains and releasing malto-oligosaccharides, they efficiently reduce staling by lowering the rate of amylopectin retrogradation without disadvantageous side effects caused by excessive weakening of the amylose network.

To our knowledge, maltotetraohydrolase (EC 3.2.1.60) from *P. saccharophila/stutzeri* has only been used in baking via two Danisco products which were pre-cursors to this G4-amylase. Closely related maltotetraohydrolases (EC 3.2.1 60) from *P. stutzeri* strains have been used for producing maltotetraose and maltotetraose syrups marketed for use in foods since the 1980s[25]. Hayashibara Company Ltd markets a maltotetraose syrup called Tetrup which is produced using maltotetraohydrolase (http://www.hayashibara.co.jp/eng/contents_pdf.html) and holds patent EP289138B1 on maltotetraose-producing amylase from e.g. *P. stutzeri* and its use for maltotetraose production.

Maltotetraohydrolase (EC 3.2.1.60) belongs, like fungal α-amylase from *A. oryzae* (EC 3.2.1.1) and maltogenic amylase (EC 3 2.1.133), to the so-called α-amylase family or glycoside hydrolase family 13. It is a large enzyme family constituting many different reactions and product specificities (http://afmb.cnrs-mrs.fr/CAZY/) and sharing a common $(\beta/\alpha)_8$- or TIM-

barrel catalytic domain. It consists of a closed eight-stranded parallel ß-sheet surrounded by eight α-helices. Four short conserved sequence motifs with seven residues strictly conserved in the α-amylase family are involved in forming the active site at the C-terminal ends of the ß-strands in the $(ß/\alpha)_8$-barrel. These four conserved sequence regions of the α-amylase family enzymes contain the three conserved catalytic residues Asp, Glu and Asp.

CAZy Family	Glycoside Hydrolase Family 13
Known Activities	α-amylase (EC 3.2.1.1); pullulanase (EC 3.2.1.41); cyclomaltodextrin glucanotransferase (EC 2.4.1.19); cyclomaltodextrinase (EC 3.2.1.54); trehalose-6-phosphate hydrolase (EC 3.2.1.93), oligo-α-glucosidase (EC 3.2.1.10); maltogenic amylase (EC 3.2.1.133); neopullulanase (EC 3.2.1.135); α-glucosidase (EC 3.2.1.20); maltotetraose-forming α-amylase (EC 3.2.1.60); isoamylase (EC 3.2.1.68); glucodextranase (EC 3.2.1.70), maltohexaose-forming α-amylase (EC 3.2.1.98); branching enzyme (EC 2.4.1.18); trehalose synthase (EC 5.4.99.16); 4-α-glucanotransferase (EC 2.4.1.25); maltopentaose-forming α-amylase (EC 3.2.1.-) ; amylosucrase (EC 2.4.1.4) ; sucrose phosphorylase (EC 2.4.1.7); malto-oligosyltrehalose trehalohydrolase (EC 2.4.1.141); isomaltulose synthase (EC 5.4.99.11).
Mechanism	Retaining
Catalytic Nucleophile/Base	Asp (experimental)
Catalytic Proton Donor	Glu (experimental)
3D Structure Status	Available (see PDB) Fold $(\beta/\alpha)_8$

The enzyme preparation will be used in bakery products such as bread, bread buns, tortillas and crackers to delay the staling of these bakery products.

6.2 Use Levels

To obtain the desired anti-staling effects of this G4-amylase, the recommended dose is 2.0-30.0 mg enzyme protein/kg flour.

6.3 Enzyme Residues in the Final Foods

The enzyme is largely inactivated during baking and has no further technical effect after baking

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

The safety of the production organism must be the prime consideration in assessing the safety of an enzyme preparation intended for use in food. [3] If the organism is non-toxigenic and non-pathogenic, then it is assumed that foods or food ingredients produced from the organism, using current Good Manufacturing Practices, are safe to consume.[4] Pariza and Foster[3] define a non-toxigenic organism as 'one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure' and a non-pathogenic organism as 'one that is very unlikely to produce disease under ordinary circumstances.' *B. licheniformis* meets these criteria for non-toxigenicity and non-pathogenicity

7.1.1 Safety of the host

Bacillus licheniformis is considered a Class 1 Containment Agent under the NIH Guidelines for Research Involving Recombinant DNA Molecules and by the Ministerie van de Vlaamse Gemeenschap in Flanders, Belgium, and is also considered as GILSP worldwide.

Mixed carbohydrase and protease from *B. licheniformis* were affirmed as GRAS by FDA on January 4, 1983 (48 FR 239). Also the FDA has no questions to four GRAS notices on enzymes derived from *B. licheniformis*:

- Alpha-amylase derived from *B. licheniformis* carrying a gene encoding a modified alpha-amylase derived from *B licheniformis* and *Bacillus amyloliquefaciens* (GRN No. 22),
- Alpha-Amylase derived from *Bacillus licheniformis* carrying a gene encoding alpha-amylase from *B. stearothermophilus* (GRN No. 24),
- Pullulanase derived from *B. licheniformis* carrying a gene encoding pullulanase from *Bacillus deramificans*(GRN No. 72), and
- Alpha-amylase derived from *B. licheniformis* carrying a gene encoding a modified alpha-amylase from *B licheniformis* (GRN No. 79).

Amylase from *B. licheniformis* has been reviewed by the Joint Expert Committee on Food Additives (JECFA) of FAO/WHO and an acceptable daily intake (ADI) "not specified" has been set[26]

B. licheniformis, including genetically modified strains, has been approved for the production of amylase enzymes in the food industry in France and it is also approved for the production of proteases and pullulanase (Arrêté du 05/09/1989 et compléments).

Strains of *B. licheniformis* are found in Table V of Division 16 of "Canadian Food and Drugs Act and Regulations", as an authorized source for amylases and proteases in several food applications.

The species *B. licheniformis* is an accepted source of safe food enzymes in the literature. The safety of *B. licheniformis* strains was recently reviewed by Priest *et al*[27]. It is also recognized as a safe host strain for recombinantly derived enzymes by Olempska-Beer *et al*[28].

Pathogenic strains of *B. licheniformis* are NOT described in the Bergey Manual[10] or in the ATCC and other catalogues. The species *B. licheniformis* does not appear on the Proposal for a Council Directive amending the "Directive 90/679/EEC on the protection of workers from risks related to exposure to biological agents at work".

Strains of *B. licheniformis* are found in the Sixth edition of "Food Chemicals Codex" as a source for the enzymatic preparation of carbohydrase and protease used in the treatment of foods.

The parent strain of the current production strain and its progeny, *B. licheniformis* BRA7, have been used by Genencor for the production of α-amylase enzyme preparations since 1989, as well as for the production of proteases, pullulanase and xylanase

Numerous oral toxicity mutagenicity and carcinogenicity studies using enzyme products from *B. licheniformis* BRA7-derived strains have been performed, and no evidence of a toxic or mutagenic effect has been observed.

7.1.2 Safety of the donor source

See section 3.3.

7.2 Safety of the Manufacturing Process

The manufacturing process for the production of G4-amylase will be conducted in a manner similar to other food and feed production processes. It consists of a pure-culture fermentation process, cell separation, concentration, filtration and formulation, resulting in a liquid G4-amylase enzyme concentrate that is then dried and agglomerated for use in baking. The process, described in Section 4, is conducted in accordance with FDA's cGMPs as set forth in 21 C.F.R Part 110. The resultant products meet the general requirements for enzyme preparations of the FCC[23] and WHO/JEFCA[24].

7 3 Safety of G4-amylase

7.3 1 Allergenicity

According to Pariza and Foster[3], there have been no confirmed reports of allergies in consumers caused by enzymes used in food processing. Amylases have been used extensively in food processes for over 50 years and have generated no known safety concerns. It is well-known that occupational exposure to airborne flour dust and enzyme particles can lead to dermal and respiratory sensitization among industry workers (AMFEP, 1998). The rate of such occurrences is relatively low, however. Studies have shown that occupational exposure does not give rise to allergic responses among workers or consumers upon ingestion of enzyme-treated baked goods. For example, Cullinan *et al*[30] studied 17 adults with demonstrated sensitivity to *Aspergillus* species (positive skin prick response). Subjects were fed bread containing *Aspergillus*-derived α-amylase or hemicellulase and an enzyme-free control bread on consecutive days. There were no consistent clinical or functional reactions to active challenge in any of the subjects

In 1998 the Association of Manufacturers of Fermentation Enzyme Products (AMFEP) Working Group on Consumer Allergy Risk from Enzyme Residues in Food reported on an in-depth analysis of the allergenicity of enzyme products. They concluded that there are no scientific indications that small amounts of enzymes in bread and other foods can sensitize or induce allergy reactions in consumers, and that enzyme residues in bread and other foods do not represent any unacceptable risk to consumers. Further, in a recent investigation of possible oral allergencity of 19 commercial enzymes used in the food industry, there were no findings of clinical relevance even in individuals with inhalation allergies to the same enzymes, and the authors concluded "that ingestion of food enzymes in general is not considered to be a concern with regard to food allergy[31]."

7.3.2 Safety Studies

7.3.2.1 Test item

The UF Concentrate was prepared as the test item for the safety studies. UF concentrate is the ultrafiltered concentrate of the enzyme preparation, stopped during the manufacturing process before the addition of formulation ingredients. The UF concentrate is characterized by:

Enzyme activity	241318 BMU/g or 3000 BMU/mg enzyme protein
pH	4.93
Specific gravity	1.028
%TOS (100 –%ash-%moisture)	9.09%
Total Protein content	78.76 mg/ml

The UF concentrate was stored frozen in aliquots and thawed before use.

The test item was used to conduct the following studies:

Study
Acute dermal irritation study in rabbits (sequential approach)
Acute Eye Irritation/Corrosion Study in the Rabbit
Acute oral toxicity in rats – Fixed dose procedure
Sub-chronic 13 week toxicity in the rat
Bacterial Reverse Mutation Assay – Ames assay
In vitro chromosomal aberration Study.

7.3.2.2 Acute dermal irritation study in rabbits (sequential approach)

A Procedure:

The objective of this study was to assess the local irritant effect of this G4-amylase. This study was conducted according to the method recommended in the OECD Guideline No. 404 "Acute dermal irritation/corrosion" (adopted 24 April 2002) and was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999) and the requirements of Directives 2004/9/EC and 2004/10/EC.

In the initial test, the back of one rabbit was shaved and three test sites were selected. At each test site a quantity of 0.5 ml of the test material was placed under a cotton gauze patch, which was secured in position with a strip of adhesive tape. One patch was removed at each of three time points: 3 minutes, 1 hour and 4 hours after application.

In the confirmatory test, two additional rabbits were treated with 0.5 ml of the test material. One patch was applied and was allowed to remain in contact for 4

hours. The test sites were examined for irritation approximately one hour following the removal of the patches and at 24, 48 and 72 hours later

B Results

No deaths or overt signs of toxicity were observed in this study. No effects on feed consumption and weight gain were recorded. No erythema, eschar or edema was observed at these test sites at any of the examinations throughout the study.

The test material produced a primary irritation index of 0.0 and no corrosive effects were noted.

C. Evaluation

On the basis of the results obtained in this study, this G4-amylase is classified as non-irritant according to OECD Guideline No 404, "Acute Dermal Irritation/Corrosion", April 2002, and the Official Journal of the European Communities, L 110 A, volume 36, 4 May 1993 § 3.2.5 ("C", R35 or R34) and § 3 2.6.1 ("Xi", R38).

7.3.2.3 Acute Eye Irritation/Corrosion Study in the Rabbit

A. Procedure

The objective of this study was to assess the ocular irritation potential of this G4-amylase. This study was conducted according to the method recommended in the OECD Guideline No. 405 "Acute eye irritation/corrosion" (adopted 24 April 2002) and was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999) and the requirements of Directives 2004/9/EC and 2004/10/EC.

In the initial test, the test material was applied at 0.1 ml to the conjunctival sac of the right eye. The left eye served as control. Assessment of ocular damage/irritation was made at approximately 1 hour and at 24, 48 and 72 hours after application. Examination of the eye was facilitated by the use of a light source from a standard ophthalmoscope. In the confirmatory test, two additional rabbits were used.

B. Results

One rabbit was killed for humane reasons, immediately after the 48-hour observation period, due to a broken front limb. As full recovery from ocular effects had already been noted by the time of its sacrifice, the absence of this rabbit was considered not to affect the purpose or integrity of the study.

No corneal or iridial effects were noted. Minimal to moderate conjunctival irritation was noted in all treated eyes one hour after treatment. At the 24-hour observation period, only minimal conjunctival irritation was noted. All eyes were cleared by the 48 and 72-hour periods. The test material produced a maximum group mean score (PIS = primary irritation score) of 7.3/110.0 at the 1 hour observation period, a PIS of 0 7/110.0 at the 48-hour observation period and a PIS of 0.0/110.0 at the 72-hour observation period.

C Evaluation

The maximal primary eye irritation score was 7.3/110 0. According to the EEC Directive published in: EEC Directive published in: "Official Journal of the European Communities" No: L 383 A, volume 35, 29.12.1992, part B5: Acute toxicity (eye irritation) and No: L 110 A, volume 36, 04.05 1993, part 3.2.6.2 Ocular lesions (which is implemented in Commission Directive 2001/59/EC of 6 August 2001), this G4-amylase is classified as mild irritant.

7.3.2.4 Acute oral toxicity in rats – Fixed dose procedure

A. Procedure:

The objective of this study was to assess the acute toxicity of this G4-amylase when administered as a single oral dose followed by a 14-day period of observation. The information is used for both hazard assessment and ranking purposes. The study was initiated with a sighting study using 2000 mg/kg with one female Sprague Dawley CD rat. In the absence of toxicity at a dose level of 2000 mg/kg, an additional group of 4 female rats was dosed with 2000 mg/kg bw

This study was conducted according to OECD Guideline No. 420 "Acute oral toxicity – Fixed dose method" (adopted 17 December 2001) and was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999) and the requirements of Directives 2004/9/EC and 2004/10/EC.

B. Results:

No mortality was recorded in this study. There were no overt signs of systemic toxicity throughout the 14-day observation period and at necropsy

C Evaluation:

Under the conditions of this study, the oral LD_{50} was equal or greater than 2000 mg/kg bw corresponding to GHS category 4 according to Annex 3 of the OECD Guideline for Testing of Chemicals No. 420. This G4-amylase is classified as "Practically non toxic by ingestion".

7.3.2.5 A 13-week Oral (Gavage) Toxicity Study in Rats

A. Procedure

The objective of this study was to investigate the potential of this G4-amylase to induce systemic toxicity after repeated daily oral administration to Wistar rats of both sexes for 90 consecutive days. Groups of 10 rats/sex each were gavaged daily with 0 (0.9% saline), 0, 23.7, 47.4 or 79 mg total protein/kg body weight in a constant volume of 5 ml 0.9% saline/kg body weight corresponding to 0, 27.3, 54.5 or 90.9 mg TOS/kg bw/day, respectively.

Animals of the same sex were housed three or four in solid floor polypropylene cages with softwood flake bedding (Harlan UK, Oxon) and had access to water (via bottle) and feed *ad libitum*. A pelleted diet (Rodent 2014C, Harlan UK, Oxon) was used. For environmental enrichment, the animals were provided a supply of Aspen Wood Wool at each change of bedding. All groups were housed under controlled temperature, humidity and lightning conditions.

All animals were observed daily for mortality and signs of morbidity. Body weight and feed consumption were recorded weekly. Water consumption was recorded twice weekly for each cage. Ophthalmologic examination was performed on all animals prior to study initiation and in the control and high dose groups at study termination. Hematology was conducted on Day 0, 30 and 90. A functional observation battery consisting of detailed clinical observation, reactivity to handling and stimuli and motor activity examination was conducted during week 13 for all animals. Clinical chemistry was evaluated at study termination prior to necropsy on all groups. After a thorough macroscopic examination, selected organs were removed, weighed and processed for future histopathologic examination. Microscopic examination was conducted on selected organs from control and high dose animals. If a questionable finding was noted, the microscopic examination would be extended to the low and mid dose groups.

This study was conducted in accordance with OECD guideline No. 408 "Subchronic oral toxicity – Rodent: 90 day study" (adopted 21 September 1998), EPA Guideline OPPTS 870.3100 (August 1998) and was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice

Regulations 1999) and the requirements of Directives 2004/9/EC and 2004/10/EC.

B. Results

No treatment-related deaths were recorded throughout the entire investigation. One low dose female was killed *in extremis* on day 68 following signs of lethargy, pilo-erection, hunched posture and staining around the snout. These observations were considered as non-treatment related. One mid dose female was found dead on day 90 just prior to terminal kill. This animal did not show any clinical signs prior to death.

Weekly open-field arena observations did not reveal any treatment-related effects. No treatment-related changes in functional performance tests were detected. No treatment-related changes in sensory reactivities were detected in the treated groups as compared with controls. There were no biological or statistical differences between the control and treated groups with respect to body weights, feed consumption, water consumption, feed efficiency (ratio of body weight gain to dietary intake), hematology, and ophthalmologic examinations.

Females from all treated groups showed an increase in serum potassium in comparison to concurrent controls. On review of historical control data, these increases were attributed to lower than expected concurrent control values The increases in serum potassium were not statistically different from historical control values.

Females from all treated groups showed a decrease in absolute and relative ovary weights when compared to concurrent controls. On review of historical control data, the control values for this parameter were higher than the expected ranges for female rats of the age and strain employed. In the absence of histopathologic findings, these findings were not attributed to treatment. Incidental macroscopic findings were noted (one high dose male displayed small kidneys; one high dose male with hydronephrosis on the right kidney) but were not considered as treatment-related in the absence of histopathologic changes. Scattered histopathologic findings were noted, but all morphological changes were those commonly observed in rats of the age and strain employed and there were no differences in severity or incidence between control and treated groups.

C. Evaluation

There were 2 interim deaths in this study but both were considered as non-treatment related. Blood chemical changes were confined to females and consisted of an increase in serum potassium. Since the changes were within the range of the historical control data, they were not considered as treatment-related. Significant decreases in absolute and relative ovary weights were noted when

compared to concurrent control values. However, on review of historical control data, the control values for this parameter were higher than the expected ranges for female rats of the age and strain employed. Therefore, these findings were considered as non-treatment related especially in the absence of histopathological changes in the ovaries.

It can be concluded that daily administration of G4-amylase by gavage for 90 consecutive days did not result in adverse systemic toxicity or adverse effects on clinical chemistry, hematology, functional observation tests and macroscopic and histopathologic examinations. Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established at the highest dose tested, 79 mg total protein/kg bw/day corresponding to 90.0 mg TOS/kg bw/day and 241318 BMU/kg bw/day.

7.3.2.6 Bacterial Reverse Mutation Assay – Ames assay

A Procedure:

The objective of this assay was to assess the potential of G4-amylase to induce point mutations (frame-shift and base-pair) in four strains of *Salmonella typhimurium* TA 98, TA 100, TA 1535 and TA 1537 and *E. coli* strain WP2uvrA$^-$. The test material was tested both in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). The tests were performed using the plate incorporation method, at 5 dose levels, in triplicate. The dose levels selected for the assay were based on the total protein content of the test material (1 ml contained 79 mg total protein).

A preliminary toxicity test was carried out with dose levels ranging from 0.15 to 5000 µg/plate.

The test material (0.1 ml) was mixed with 0.1 ml of bacterial suspension, 2 ml of molten agar, 0.5 ml of S-9 mix or phosphate buffer and overlaying onto sterile plates of agar. Sterile distilled water served as vehicle control. After 48 hours incubation at 37°C, the plates were assessed for the number of revertant colonies and growth of the bacterial background lawn.

In the main assay, five concentrations of the test material were assayed in triplicate against each tester strain in both the presence and absence of metabolic activation. The test material (0.1 ml) was mixed with 0.1 ml of bacterial suspension, 2 ml of molten agar, 0.5 ml of S-9 mix or phosphate buffer and overlaying onto sterile plates of agar. Sterile distilled water served as vehicle control. The positive controls used for plates without S-9 mix were N-ethyl-N'-nitro-nitrosoguanidine, 9-aminoacridine and 4-nitroquinoline-1-oxide. The positive controls used for plates with S-9 mix were 2-aminoanthracene and

benzo(a)pyrene. After 48 hours incubation at 37°C, the plates were assessed for the number of revertant colonies and growth of the bacterial background lawn.

All dose levels were expressed in terms of total protein. The highest dose level tested (5000 µg/plate) is the maximum required by the OECD guideline. This assay was conducted in accordance with OECD guideline No. 471 "Bacterial reverse mutation test", the US EPA (TSCA) OPPTS harmonized guidelines, mutagenicity guidelines published by the Japanese Regulatory Authorities (METI, MHLW and MAFF) and was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999) and the requirements of Directives 2004/9/EC and 2004/10/EC.

B. Results:

In the screening assay, G4-amylase was not toxic to the test bacteria up to and including the highest dose level tested (5000 µg/plate) in both the presence and absence of S-9 mix. The test material caused no visible reduction in the growth of the bacterial background lawn at any dose level. Therefore, 5000 µg/plate was selected as the highest dose level for the main tests

In the main tests, five dose levels (50, 150, 500, 1500 and 5000 µg/plate) were used. Insoluble material was observed at and above the 1500 µg/plate but this did not prevent the scoring of the revertant colonies. No biologically or statistically significant increases in the number of revertant colonies were observed in any dose level in either main test. Statistical increases in the number of revertant colonies were noted with the positive controls in both the presence and absence of metabolic activation substantiating the sensitivity of the treat and plate assay and the efficacy of the metabolic activation mixture.

C Evaluation

Under the conditions of this assay, G4-amylase has not shown any evidence of mutagenic activity in the Ames assay.

7.3.2.7 *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes

A. Procedure

The objective of this assay was to investigate the potential of G4-amylase to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). G4-amylase was mixed with cultures of human peripheral lymphocytes both in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver; S-9 mix). This assay consisted of a preliminary toxicity (dose range finding) assay and two main tests.

Five concentrations of G4-amylase were used in the preliminary assay and at least 3 dose levels were then selected for the two main assays with the highest dose level clearly inducing a toxic effect (50% reduction in mitotic index). In the absence of cytotoxicity, the highest dose selected would be 5000 µg/ml, as recommended by the OECD guideline.

With metabolic activation:
Cells were grown in Eagle's essential medium with HEPES buffer (MEM) at 37°C with 5%CO2 in air. After 48 hours, the cell cultures were transferred to tubes and centrifuged. Approximately 9 ml of the culture medium was removed and G4-amylase (1 ml), S-9 mix (1ml), and MEM culture media (7ml) were added to the cell cultures and incubated at 37°C for 4 hours The cultures were then centrifuged, the treatment medium removed and washed with MEM culture medium. After a further centrifugation, the MEM culture medium was removed by suction and replaced with the fresh MEM culture medium. The cell cultures were then re-incubated for an additional 20 hours at 37°C in 5% CO_2 in humidified air.

Without metabolic activation.
Cells were grown in Eagle's essential medium with HEPES buffer (MEM) at 37°C with 5%CO2 in air. After 48 hours, the cell cultures were transferred to tubes and centrifuged. Approximately 9 ml of the culture medium was removed and G4-amylase (1 ml) and MEM culture media (8ml) were added to the cell cultures and incubated at 37°C for 4 hours. The cultures were then centrifuged, the treatment medium removed and washed with MEM culture medium After a further centrifugation, the MEM culture medium was removed by suction and replaced with the fresh MEM culture medium. The cell cultures were then re-incubated for an additional 20 hours at 37°C in 5% CO_2 in humidified air.

All cultures (with and without S-9 mix) were harvested 24 hours after the start of treatment. Two hours prior to harvest, Demecolcine (0.1 µg/ml) was added to all cultures to arrest all cells at the metaphase-stage of mitosis. At the harvest time, all cultures were centrifuged and the supernatant discarded. The cell pellets were resuspended in a KCl solution, incubated for 10 minutes, centrifuged and the supernatant removed. The cells were then fixed on slides, stained and scored for chromosomal aberrations

a. Cytotoxicity was evaluated using the mitotic index (number of cells in mitosis/1000 cells examined. From these results, a dose level causing a decrease in mitotic index of 50% was selected as the highest dose in the main tests.

b. Metaphase analysis (i.e evaluation of chromosomal aberration) was conducted on 100 metaphases per dose level.

c Mitomycin C and cyclophosphamide were used as positive controls for cultures without S-9 mix and with S-9 mix, respectively.

This assay was conducted in accordance with OECD guideline No. 473 "*In vitro* Mammalian chromosome aberration test" and was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999) and the requirements of Directives 2004/9/EC and 2004/10/EC.

B. Results

In the preliminary assay, the dose range was 19.53 to 5000 µg/ml. Cytotoxicity (only 6% survival) was noted at the 312.5 µg/ml dose level. Therefore, the maximum dose level selected for the 4-hour incubation period in the presence and absence of S-9 mix was 625 µg/ml and the maximum dose selected for the 24-hour continuous exposure was 312.5 µg/ml.

In both main assays, G4-amylase did not induce a statistically significant increase in the frequency of cells with aberrations either in the absence or presence of metabolic activation.

In both main assays, G4-amylase did not induce a statistically significant increase in the numbers of polyploid cells at any dose level either in the presence or absence of metabolic activation.

In both main assays, significant increases in aberrant metaphases were noted with the positive controls demonstrating the sensitivity of the tests and the efficacy of the S-9 mix.

C. Evaluation

Under the conditions of this test, G4-amylase did not induce chromosomal aberrations (both structural and numerical) in this *in* vitro cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation.

7. 4. Overall Safety Assessment and Human Exposure

According to the Directive of the Commission 93/21/EEC of April 27, 1993, G4-amylase is non hazardous based on acute oral and irritation studies. In genotoxicity studies, G4-amylase is not mutagenic, clastogenic or aneugenic. Daily oral administration of G4-amylase up to and

including a dose level of 79 mg total protein/kg bw/day (90.0 mg TOS/kg bw/day) does not result in any manifestation of systemic, hematologic, functional or histopathologic adverse effects.

7.4.1 Identification of the NOAEL

In the 90-day oral (gavage) study in rats (SafePharm Lab No. 2420-0008), a NOAEL was established at 79 mg total protein/kg bw/day (equivalent to 90.0 mg TOS/kg bw/day). The study was conducted in compliance with both the UK and OECD Good Laboratory Practice Regulations and was designed based on OECD guideline No. 408. Since human exposure to G4-amylase is through oral ingestion, selection of this NOAEL is thus appropriate.

No Observed Adverse Effect Level = 79 mg total protein/kg bw/day
= 90.0 mg TOS/kg bw/day

7.4.2 Human Exposure Assessment

G4-amylase is used in bakery. Based on the Food Consumption Statistics collected from 1979-1988 by OECD (1991), Italy has the highest daily consumption of bread, i.e 4.92 g/kg bw/day. Data from the USDA, Agricultural Research Service (1997) reveals the maximum daily bread consumption in the US as 4.07 g/kg bw/day. Therefore, for the purpose of this safety assessment:

1. The maximum daily bread consumption of 4.92 g/kg bw/day is used to represent a worst-case scenario:
   Maximum daily bread consumption = 4.92 g/kg bw/day;
2. Since the flour content in bread is 66%, the maximum daily flour consumption is:
   4.92 g/kg bw/d X 66% = 3 25 g/kg bw/day;
3. G4-amylase is applied at the recommended range of 2.0 to 30.0 mg enzyme protein/kg flour;
4. Maximum intake of G4-amylase from flour:
   3.25 g/kg bw/day X 30 mg/kg flour = 0.098 mg enzyme protein/kg bw/day;
5. Daily exposure to G4-amylase (worst case) assuming that 100% of enzyme remains in the bread, not removed or inactivated by heat and also on the assumption of 100% market penetration =
   0.098 mg enzyme protein/kg bw/day.

Risk Assessment

Risk for humans through ingestion of bakery made from flour treated with G4-amylase is measured by a Margin of Exposure (MOE), which determines how close the estimated daily human exposure comes to a No Observable Adverse Effect Level (NOAEL). Generally, a MOE greater than 100 means consumption is not of concern.

$$\text{Margin of exposure} = \frac{\text{No observed adverse effect level}}{\text{Daily exposure}}$$

Based on the results from the 90-day oral (gavage) feeding study cited above,

$$\text{Margin of exposure} = \frac{\text{79 mg enzyme protein/kg bw/day}}{\text{0.098 mg/kg bw/day}} = 806.$$

7 5 Conclusion

The safety of G4-amylase is assessed in a battery of toxicology studies investigating its acute oral, irritation, mutagenic and systemic toxicity potential. G4-amylase is not a skin irritant, is not acutely toxic by ingestion and is a mild eye irritant. A battery of genotoxicity assays was conducted and under the conditions of these assays G4-amylase is not a mutagen, a clastogen, or an aneugen.

Daily administration of G4-amylase by gavage for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at 79 mg total protein/kg bw/day (equivalent to 90.0 mg TOS/kg bw/day).

Even under the worst case scenario that G4-amylase is applied at the maximum rate (30 mg enzyme protein/kg flour) and the enzyme is not destroyed and/or removed during baking, the use of G4-amylase as a food additive in bakery is not expected to result in adverse effects to humans. Based on the NOAEL of 79.0 mg total protein/kg bw/day (90.0 mg TOS/kg bw/day), a safety margin of 806-fold exists for the proposed uses

8. BASIS FOR GENERAL RECOGNITION OF SAFETY

As noted in the Safety sections above, enzyme preparations derived from *B. licheniformis*, including α-amylase, protease, pullulanase, and xylanase, are well recognized by qualified experts as being safe. Published literature, government laws and regulations, reviews by expert panels such as FAO/WHO JECFA, as well as Genencor's own unpublished safety studies, support such a conclusion.

Bacillus licheniformis is widely used by enzyme manufacturers around the world for the production of enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. It is a known safe host for enzyme production. An enzyme preparation derived from a recombinant microorganism will be safe if the host microorganism is nonpathogenic and non-toxigenic; the genetic information that is introduced into the host microorganism is well characterized; and the added DNA does not encode and express any known harmful or toxic substances. Information is provided in this document to support that the components of the enzyme preparation meet these standards.

The safety studies conducted by Genencor established a NOAEL of 79.0 mg total protein/kg bw/day (90.0 mg TOS/kg bw/day) and a safety margin of 806-fold for the proposed uses.

Utilizing the information provided, the enzyme preparation was taken through the Pariza-Johnson decision tree for microbial enzymes (see Appendix 3) and the preparation was determined to be acceptable for use in foods as described above.

Based on the available data from the literature and generated by Genencor, the company has concluded that the enzyme preparation from *B. licheniformis* strain GICC03279 is safe and suitable for use in bakery products.

In addition, the safety determination including construction of the production organism, the production process and materials, and safety of the product were reviewed by Dr. Michael W. Pariza, who concurred with the company's conclusion that the product is GRAS (see Appendix 4) (note, the safety studies on this G4-amylase were conducted after the GRAS determination by Dr. Pariza to support regulatory approvals outside the US where toxicology testing is required).

GENENCOR

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10. LIST OF APPENDICES

Appendix 1 – The amino acid sequence of the G4-amylase

Appendix 2 - The Manufacturing process

Appendix 3 – The Pariza/Johnson Decision Tree

Appendix 4 – GRAS Panel Report

1

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Appendix 1 – The amino acid sequence of this G4-amylase

Amino acid sequence alignment of this G4-amylase vs. PS4 wild type Maltotetraohydrolase. The amino acids residue positions changed between wild type PS4 and this G4-amylase are shown in green.

```
PS4 MATURE       (     1) -dqagkspagvryhggdeilqgfhwnvvreapndwynilrqqastiaad
This G4 MATURE   (     1)   . .     .......................└┘..............

PS4 MATURE       (    50) gfsaiwmpvpwrdfsswtdggksgggegyfwhdfnkngrygsdaqlrqaa
This G4 MATURE   (    51) ................... └┘   ...................  ..

PS4 MATURE       (   100) galggagvkvlydvvpnhmnrgypdkeinlpagqgfwrndcadpgnypnd
This G4 MATURE   (   101) ............ ...     └┘     .   . .└┘....  └┘   └┘

PS4 MATURE       (   150) cddgdrfiggesdlntghpqiygmfrdelanlrsgygaggfrfdfvrgya
This G4 MATURE   (   151) .... . └┘...└┘         .. .   └┘        .  ..  ......

PS4 MATURE       (   200) pervdswmsdsadssfcvgelwkgpseypswdwrntaswqqiikdwsdra
This G4 MATURE   ( 201)       .. .       ......└┘.... └┘ . .     ...........

PS4 MATURE       (   250) kcpvfdfalkermqngsvadwkhglngnpdprwrevavtfvdnhdtgysp
This G4 MATURE   (   251)  .... .   . ................................ ...

PS4 MATURE       (   300) gqnggqhhwalqdglirqayayiltspgtpvvywshmydwgygdfirqli
This G4 MATURE   (   301) .......└┘.└┘..........    . ........└┘.. .          .

PS4 MATURE       (   350) qvrrtagvradsaisfhsgysglvatvsgsqqtlvvalnsdlanpgqvas
This G4 MATURE   (   351) ............ .                              .......

PS4 MATURE       (   400) gsfseavnasngqvrvwrsgsgdgggndggegglvnvnfrcdngvtqmgd
This G4 MATURE   (   401) .        .        . ......     --------------------

PS4 MATURE       (   450) svyavgnvsqlgnwspasavrltdtssyptwkgsialpdgqnvewkclir
This G4 MATURE   (   450) --------------------------------------------------

PS4 MATURE       (   500) neadatlvrqwqsggnnqvqaaagastsgsf
This G4 MATURE   (   500) --------------------------------
```

2

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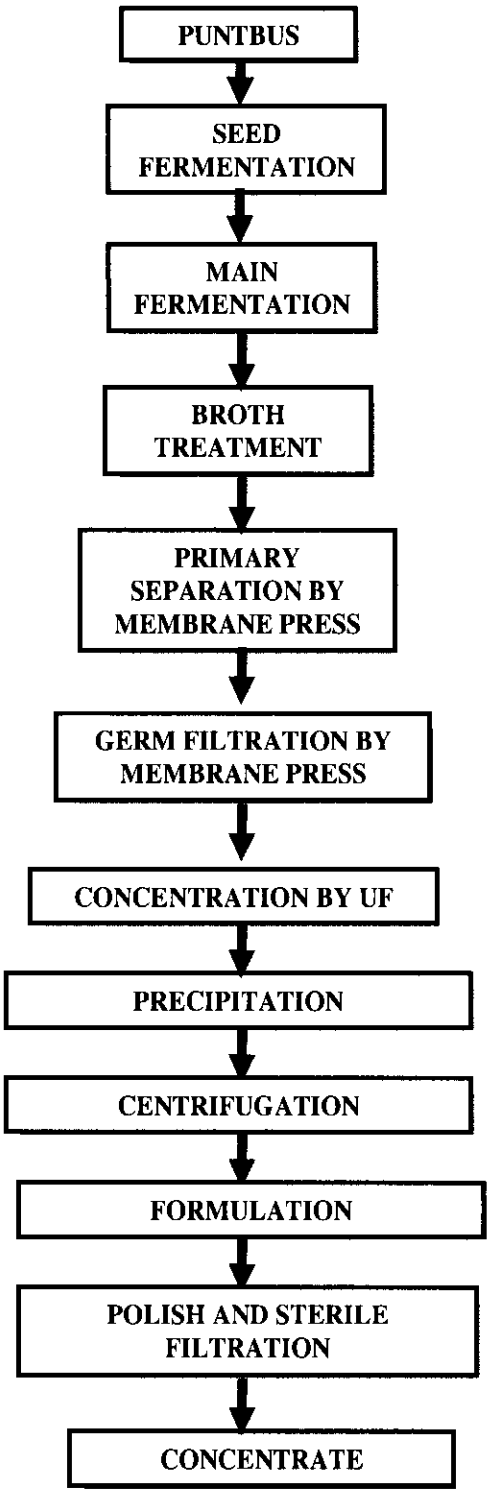
Appendix 2- The Manufacturing Process



3

Appendix 3 – Analysis of Safety Based on Pariza/Johnson Decision Tree

Pariza and Johnson have published guidelines for the safety assessment of microbial enzyme preparations (2001). These guidelines are based upon decades of experience in the production, use and safety evaluation of enzyme preparations. The safety assessment of a given enzyme preparation is based upon an evaluation of the toxigenic potential of the production organism. The responses below follow the pathway indicated in the decision tree The outcome of this inquiry is that this G4-amylase enzyme preparation is "ACCEPTED" as safe for its intended use.

1. **Is the production strain genetically modified?** Yes, go to 2

2. **Is the production strain modified using rDNA techniques?** Yes. The donor of this G4-amylase gene is *Pseudomonas saccharophila* and the host is *Bacillus licheniformis*. Go to 3a.

3a. **Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food?** Yes. Three previous generations of the enzyme, produced in *Bacillus subtilis*, have been determined to be GRAS. Furthermore, amylases with the designation 3.2.1.1, which are produced from fungal, bacterial and cereal sources, are widely used in the food industry and have been so for decades. Maltotetraohydrolases (EC 3.2.1.60) from *Pseudomonas stutzeri* strains, which are closely related to the three generations of this G4-amylase, have been used for producing maltotetraose and malto-tetraose syrups for use in foods since the 1980s[25, 32,-33]. Maltotetraose-forming amylase from *P. stutzeri* is listed as a natural additive in the Korean Food Additives Code. Go to 3c

3c. **Is the test article free of transferable antibiotic resistance gene DNA?** Yes. Transformable DNA was not detected at or above the limit of 5 ng/ml in the enzyme preparation manufactured using this host and production process. Go to 3e.

3e. **Is all other introduced DNA well characterised and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?** Yes. The only DNA introduced is the endogenous *B. licheniformis* cat gene and this G4-amylase gene. The inserted sequences do not code for any known toxins. Go to 4

4. **Is the introduced DNA randomly integrated into the chromosome?** No. The construct is designed to integrate into the *cat* locus by Campbell type recombination Go to 5.

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

was employed? Yes. Given the targeted integration (see 4 above) there is no concern for pleiotropic effects. Go to 6.

6. **Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?** Yes. The *B. licheniformis* host and methods of integration of the enzyme gene have been used by Genencor for production of many enzyme production organisms. Toxicology testing on 5 products from this strain lineage confirms the safety of the lineage. **Accept.**

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Appendix 4 – GRAS Panel Report

GENENCOR

Michael W. Pariza Consulting, LLC
7102 Valhalla Trail
Madison, WI 53719

Michael W Pariza, Member

March 7, 2008

Alice J. Caddow
Vice President, Regulatory
And Environmental Affairs
Genencor, A Danisco Division
925 Page Mill Road,
Palo Alto, CA 94304

Dear Ms. Caddow:

I have reviewed the information you provided on Danisco's alpha-amylase SAS3 enzyme preparation expressed in a genetically modified strain of *Bacillus licheniformis* Bra 7 In this evaluation, I considered the biology and the safe lineage of the genetically-modified *Bacillus licheniformis* Bra 7 production organism, information provided by Danisco on the safety of the modified gene for alpha-amylase SAS3 and its expression product (the alpha-amylase SAS3 protein), and information regarding the gene source organism which has been reclassified as *Pseudomonas stutzeri* IAM1504 based on 16s rDNA sequence analysis (it had been previously misclassified as *P. saccharophilia* IAM1504 based on older biochemical/physiological assessment methodology).

Alpha-amylase enzyme preparations designated D34 and SAS2 that are substantially equivalent to SAS3 were previously determined to be GRAS by scientific procedures, as was SAS3 expressed in *B. subtilis*. The genes for D34 and SAS2 were both derived from *Pseudomonas stutzeri* IAM1504 and cloned into a strain of *Bacillus subtilis* that has a long history of safe use in food enzyme manufacture. SAS3 differs from the SAS2, D34, and *Pseudomonas stutzeri* IAM1504 wild-type amylase (designated PS4wt) catalytic core, by the number of amino acid modifications introduced into the amylase gene These mutations were introduced to enhance stability and catalytic activity, thereby improving enzyme performance in baking applications.

The *B. licheniformis* Bra 7 production organism, which has been genetically modified to

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express the alpha-amylase SAS3 gene, has a long history of safe use in food enzyme manufacture. It was derived from a *B. licheniformis* safe strain lineage that has been previously described in GRAS assessment documents. These documents were reviewed by expert scientific panels, and determined to be GRAS by scientific procedures for food enzyme manufacture

Based on these considerations, I conclude that the Danisco alpha-amylase SAS3 enzyme preparation derived from a genetically modified strain of *Bacillus licheniformis* Bra 7 by the manufacturing procedure you have described is GRAS for use in baking applications under the intended conditions of use that you have described.

Please note that this is a professional opinion directed at safety considerations only and not an endorsement, warranty, or recommendation regarding the possible use of the subject product by you or others.

Sincerely,

Michael W. Pariza
Wisconsin Distinguished Professor of
Food Microbiology and Toxicology
Member, Michael W. Pariza Consulting LLC

SUBMISSION END

RECEIVED
FEB 2009
BY: (b)(6)

GENENCOR
A Danisco Division

AM

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February 4, 2009

Dr. Robert Martin
Office of Food Additive Safety, HFS-255
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

RE: GRSN on G4-amylase, submitted January 8, 2009

Dear Dr. Martin:

Please accept the enclosed amendment to the above referenced GRAS Notice.

If you have questions or require additional information, please contact me at 650-846-7557 or fax at 650-845-6505.

Sincerely,

(b)(6)

Alice J. Caddow
Vice President of Regulatory
And Environmental Affairs

Enclosures (3 binders)

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FEB 2009
BY: (b)(6)

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G4-amylase Enzyme Preparation from *Bacillus licheniformis* Expressing a Modified Maltotetraohydrolase (G4-amylase) Gene from *Pseudomonas saccharophila* Is Generally Recognized As Safe For Use in Food Processing

Notification Submitted by Genencor, a Danisco Division

January 7, 2009
(Amended February 4, 2009)

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1. GENERAL INTRODUCTION

The G4-amylase enzyme preparation under consideration is produced by submerged fermentation of *Bacillus licheniformis* carrying the gene encoding a protein engineered variant of the maltotetraohydrolase enzyme from *Pseudomonas saccharophila*. The *mta* gene encoding the native maltotetraohydrolase PS4wt from *P. saccharophila* was cloned by PCR based on the mta sequence[1]. To avoid heterogeneity of the G4-amylase upon limited proteolysis, the starch-binding domain was removed by introducing a stop codon after residue G429.

Subsequently, this G4-amylase has been developed by successive cycles of random, site specific mutagenesis and site scanning mutagenesis combined with screening for thermostability and exo-specificity. This G4-amylase contains sixteen amino acid changes compared to the sequence of the catalytic core of PS4wt.

By introducing these mutations, the temperature stability and baking performance of the enzyme is increased, making this G4-amylase far better suited for anti-staling applications than the wild-type maltotetraohydrolase enzyme from *P. saccharophila*.

This G4-amylase will be used to delay the staling process in bread and other baked goods and thereby extend the period for which the products have an acceptable eating quality. This is a benefit to the bread producers and to consumers, as bread will keep fresh for a longer time and disposal of stale bread and baked goods is minimized. The ability of this G4-amylase to form maltotetraose can also be exploited in the starch processing industry. In these applications the enzyme is considered a processing aid.

The systematic name of the principle enzyme activity is 4-α-D-Glucan maltotetraohydrolase. Other names used are glucan 1,4-α-maltotetraohydrolase, exo-maltotetraohydrolase, maltotetraohydrolase, maltotetraose-forming amylase, and G4-amylase.

The enzyme catalyses hydrolysis of 1,4-α-D-glucosidic linkages in amylaceous polysaccharides to remove successive maltotetraose residues from the non-reducing chain ends.

The EC number of the enzyme is 3.2.1.60, and the CAS number is 37288-44-1.

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. 170.36, Danisco has determined that its G4-amylase enzyme preparation from a modified strain of *B. licheniformis* expressing a protein engineered maltotetraohydrolase enzyme from *P. saccharophila* is a Generally Recognized As Safe ("GRAS") substance for the intended food application and is, therefore, exempt from the requirement for premarket approval. The information provided in the following sections is the basis of our determination of GRAS status of this G4-amylase enzyme preparation produced by a *B. licheniformis* host, which has been modified to express a gene encoding a protein engineered maltotetraohydrolase enzyme from *P. saccharophila*.

Our safety evaluation in Section 7 includes an evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure to the preparation.

The safety of the production organism must be the prime consideration in assessing the safety of an enzyme preparation intended for food use.[2,3] The safety of the production organism for this G4-amylase, *B. licheniformis,* is discussed in Sections 2 and 7. Another essential aspect of the safety evaluation of enzymes derived from genetically modified microorganisms is the identification and characterization of the inserted genetic material.[4-9] The genetic modifications used to construct this production organism are well defined and are described in Section 2. The safety evaluation described in Section 7 shows no evidence to indicate that any of the cloned DNA sequences and incorporated DNA code for or express a harmful toxic substance.

1.1 Name and Address of Notifier

Genencor, A Danisco Division
925 Page Mill Road
Palo Alto, CA 94304

1.2 Common or Usual Name of Substance

G4-amylase enzyme preparation from *B. licheniformis* expressing the gene encoding a protein engineered variant of the maltotetraohydrolase enzyme from *Pseudomonas saccharophila.*

1.3 Applicable Conditions of Use

This G4-amylase will be used to delay the staling process in bread and other baked goods and thereby extend the period for which the products have an acceptable eating quality. This is a benefit to the bread producers and to consumers as bread will keep fresh for a longer time and disposal of stale bread and baked good is minimized. The ability of this G4-amylase to form maltotetraose can also be exploited in the starch processing industry. In these applications the enzyme is considered a processing aid.

1.4 Basis for GRAS Determination

This GRAS determination is based upon scientific procedures.

1.5 Availability of Information for FDA Review

A notification package providing a summary of the information which supports this GRAS determination is enclosed with this notice. The package includes a safety evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure. Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying upon request.

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The production organism of the G4-amylase enzyme preparation is *B. licheniformis* strain GICC03279. It is derived by recombinant DNA methods from a strain of *B. licheniformis* modified to express a modified G4-amylase gene DNA (see Section 2.2). *Bacillus licheniformis* is considered a Class 1 Containment Agent under the NIH Guidelines for Research Involving Recombinant DNA Molecules and by the Ministerie van de Vlaamse Gemeenschap in Flanders, Belgium, and is also considered as GILSP worldwide. It also meets the criteria for a safe

production microorganism as described by Pariza and Johnson[2] and several expert groups.[3-9] It contains the modified G4-amylase gene under the regulation of a native *B. licheniformis* promoter and terminator along with a selectable marker, the native *B. licheniformis cat* gene.

The inserted DNA was integrated into the host on a in a vector derived from *Bacillus* plasmids pUB110 and pE194. The introduced DNA integrated into the Bacillus chromosome at the *cat* locus by Campbell type recombination. After integration all vector sequences of the plasmid were deleted by recombination between direct repeated *cat* sequences.

2.2 Host Microorganism

The host organism is *B. licheniformis* BRA7. *B. licheniformis* BRA7 was modified through deletion of several enzyme activities (proteases, amylase), a sporulation gene and the native chloramphenicol resistance genes to make it suitable for expression of G4-amylase. This strain lineage has been used by Genencor as a host for the commercial production of a number of α-amylases for the starch liquefaction business since 1989, as well as for production of protease, pullulanase, xylanase, and glycerophospholipid cholesterol acyltransferase. The strain has a sporulation frequency of less than 10^{-8} as determined by comparing the titer of the colony forming units (CFU) in the culture before and after heating at 85° C for 10 minutes. The strain, which has a history of safe large-scale fermentation, has been typed as *B. licheniformis* based on 16S rDNA gene sequencing and ribotyping.

2.3 G4-amylase Expression Vector

The vector used in this construction contains the following features: a temperature sensitive origin of replication (*ori* pE194, for replication in *Bacillus*), *ori* pBR322 (for amplification in *Escherichia coli*), the pUB110 neomycin resistance gene (*neo*) for initial selection, and the native *B. licheniformis* chloramphenicol resistance gene (*cat*) for selection, chromosomal integration and cassette amplification. The *cat* gene is present on a larger native *B. licheniformis* chromosomal fragment surrounding the coding sequence with upstream and downstream sequences. Part of the upstream *cat* sequence (called 5' repeat) is present twice on the plasmid to allow amplification of the expression cassette on the chromosome. The expression cassette (containing a native promoter and signal sequence and the G4-amylase gene) is located between these repeats.

The genetic construction was evaluated at every step to assess the incorporation of the desired functional genetic information and the final construct was verified by Southern blot analysis to confirm that only the intended genetic modifications to the *B. licheniformis* strain had been made.

2.4 Stability of the Introduced Genetic Sequences

The production strain proved to be 100% stable after at least 60 generations of fermentation, judged by chloramphenicol resistance and G4-amylase production.

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2.5 Antibiotic Resistance Gene

The construction of the production organism utilized the native chloramphenicol acetyltransferase (*cat*) gene from *B. licheniformis* conferring chloramphenicol resistance; no new antibiotic resistance was introduced.

2.6 Absence of Production Microorganism in Product

The absence of the production microorganism is an established specification for the commercial product at a detection limit of 1CFU/g. The production organism does not end up in food and therefore, the first step in the safety assessment as described by the IFBC[4] is satisfactorily addressed.

3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1 Enzyme Identity

Classification	Glycoside hydrolase
IUBMB Nomenclature	4-α-D-Glucan maltotetraohydrolase
IUBMB Number:	3.2.1.60
CAS Number:	37288-44-1
Reaction catalyzed	hydrolysis of 1,4-α-D-glucosidic linkages in amylaceous polysaccharides to remove successive maltotetraose residues from the non-reducing chain ends.

3.2 Amino Acid Sequence

The G4-amylase in question is a variant of the wild type maltotetraohydrolase (PS4wt) from *P. saccharophila* strain IAM 1504 (obtained from the Institute of Applied Microbiology (IAM) Culture Collection, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Yayoi, Bunkyo-Ku, Tokyo 113-0032, Japan). Compared to the wild type enzyme PS4wt encoded by the gene *mta* of *P. saccharophila* strain IAM 1504[1], the starch-binding domain has been removed and 16 out of the remaining 429 amino acids of the catalytic core have been changed in order to improve thermostability and baking performance of the enzyme.

The amino acid sequence of this G4-amylase is shown in Appendix 1.

3.3 G4-amylase Donor Safety

Pseudomonas saccharophila and *Pseudomonas stutzeri* are gram negative, rod shaped bacteria mobile by means of a single polar flagellum. They are obligate aerobes, except in media with nitrate. They do not require organic growth factors and are found in soil and water[10].

Even though it is only the gene encoding the maltotetraose forming amylase which is transferred from the donor organism to the host organism, the safety of the donor organism was assessed, as *P. stutzeri* and *P. saccharophila* do not have a long history of safe use in the food industry. The safety was assessed based on search in the scientific literature.

The databases Medline, Chemtox and Chemical Abstract were searched. As the donor organism was identified as *P. saccharophila* by IAM, but as *P. stutzeri* based on 16s rDNA analysis, both the words "Pseudomonas" and "stutzeri or saccharophila" were used. These were combined with "pathogen or patogen or allergen or allergic or allergy or toxin or toxic or toxicology" for the period 1980 to 26 June 2006.

Reisler and Blumberg[11] reviewed the literature in the English language in the period January 1966 through August 1998 and reported 29 cases of *P. stutzeri* infections in humans. Among these cases were 2 deaths. Both patients were immunocompromised and in one case the death may not be directly related to the initial infection. They reported one case of community-acquired vertebral osteomyelitis caused by *P. stutzeri* and described it to be the first recorded case of a community-acquired, atraumatic infection in a previously healthy, non-immunocompromised person. The patient was successfully treated with antibiotics.

The database search revealed 3 other published papers on human infection cases with *P. stutzeri* (Campos-Herrero *et al*[12]; Potvliege *et al*[13] Puzenat *et al*[14]. All 3 patients were cured for their *P. stutzeri* infection by antibiotics. Two of the cases were included in the Reisler and Blumberg review, while one was reported more recently.

Lin *et al*[15] reported the isolation of *P. stutzeri* from the respiratory tract or bone marrow of dead chickens amongst 12 other Pseudomonas species and two other gram-negative hemolytic bacteria. Of the 15 species, 7 caused more than 50% mortality in chickens in an experimental set-up, while *P. stutzeri* resulted in less than 30% mortality. All strains including *P. stutzeri* were susceptible to some antibiotics and resistant to others.

Cortese *et al*[16] reported that *P. stutzeri* strain KC contains the MoeZ gene, which is essential for the synthesis of pyridine-2,6-bis(thiocarboxylic acid) (pbtc), a potential pathogenicity factor. However, other *P. stutzeri* strains and other Pseudomonas species tested lacked the pbtc synthesis genes. Cortese *et al*[17] showed that *P. stutzeri* strain KC and *P. putida* DSM301 are able to produce pbtc, which is involved in the overall level of iron uptake, but they do not present any correlation to potential pathogenicity. Sebat *et al*[18] also describes *P. stutzeri* strain KC as a pbtc-forming organism and the strain demonstrated antagonism towards *E. coli*.

Martins *et al*[19] reports that *P. stutzeri* in some papers has been reported as capable of producing paralytic shellfish toxins (PSTs), but that results of autonomous (not related to presence of dinoflagellates) production is controversial. From their results in this paper the researchers do not conclude that autonomous production of PSTs by Pseudomonas can be excluded, but they show that utmost care must be taken in interpreting results and that previous findings have been false positives. Baker *et al*[29] reports that they can not rule out the possibility that *P. stutzeri* strain SF/PS produces saxitoxins; however, their data clearly demonstrate that the strain accumulates at

least five different fluorescent compounds that could be easily mistaken for the saxitoxin derivative GTX_4.

4. MANUFACTURING PROCESS

This section describes the manufacturing process for this G4-amylase which follows standard industry practice.[20-22] For a diagram of the manufacturing process, see Appendix 2. The quality management system used in the manufacturing process complies with the requirements of ISO 9001. The enzyme preparation is also manufactured in accordance with FDA's current Good Manufacturing Practices ("cGMP") as set forth in 21 C.F.R. Part 110.

4.1 Raw Materials

The raw materials used in the fermentation and recovery process for this G4-amylase concentrate are standard ingredients used in the enzyme industry.[20-22] All the raw materials conform to the specifications of the Food Chemicals Codex, 6th edition, 2008 ("FCC"),[23] except for those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal requirements have been made in line with FCC requirements and acceptability of use for food enzyme production. Danisco uses a supplier quality program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and are verified upon receipt.

The antifoam and flocculants used in the fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 24, 1998. The maximum use level of these antifoam in the production process is $\leq$0.15% and of the flocculants <1.5%.

4.2 Fermentation Process

The G4-amylase is manufactured by submerged straight-batch or fed-batch pure culture fermentation of the genetically modified strain of *B. licheniformis* described in Section 2. All equipment is carefully designed, constructed, operated, cleaned and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are conducted periodically to ensure absence of foreign microorganisms and confirm production strain identity.

4.2.1 Production organism

A new lyophilized stock culture vial of the *B. licheniformis* production organism described in Section 2 is used to initiate the production of each batch. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

4.2.2 Criteria for the rejection of fermentation batches

Growth characteristics during fermentation are observed microscopically. Samples are taken from each fermentation stage (inoculum, seed and main fermenter) before inoculation, at regular intervals during growth and before harvest or transfer. These samples are tested for microbiological contamination by plating on a nutrient medium.

A fermentation batch is declared as 'contaminated' if colony forming units (CFU) of bacteria or fungi other than the production strain are present at levels $>10^3$CFU/ml.

If a fermentation batch is determined to be contaminated, it will be rejected if deemed appropriate. If the contamination is minor and determined to be from common non-pathogenic environmental microbes, the fermentation may be processed.

4.3 Recovery Process

The recovery process is a multi-step operation which starts immediately after the fermentation process and consists of both concentration and formulation processes.

4.3.1 Concentration process

The enzyme is recovered from the culture broth by the following series of operations:

1. Primary separation –centrifugation or filtration
2. Polish filtration – for removal of residual production strain organisms and as a general precaution against microbial degradation
3. Concentration – ultrafiltration
4. Precipitation
5. Centrifugation
6. Formulation
7. Polish and sterile filtration
8. Concentration.

4.3.2 Formulation and standardization process

The ultrafiltered concentrate is stabilized with sodium benzoate. The preserved ultrafiltered concentrate is then dried and agglomerated using any one of the common drying methods, such as spray drying, fluid bed agglomeration or fluid bed spray drier.

4.3.3 Quality control of finished product

The final G4-amylase liquid concentrate from *B. licheniformis* is analyzed in accordance with the general specifications for enzyme preparations used in food processing as

established by the Joint FAO/WHO Expert Committee on Food Additives ("JEFCA")[24] in 2006 and the FCC[23]. These specifications are set forth in Section 5.

The dried product is tested for enzyme activity, Total Viable Count and Dry Matter. The other assays conducted on the liquid concentrate are not repeated for the dried product. The dried product also is in compliance with the general specifications for enzyme preparations used in food processing as established by JEFCA[24] in 2006 and the FCC[23].

5. COMPOSITION AND SPECIFICATIONS

5.1 Quantitative Composition

The liquid concentrate is stabilized with 1% potassium sorbate and tested to demonstrate that it meets the specifications in section 5.2.

The liquid concentrate is then mixed with microcrystalline cellulose and spray dried to produce a product which is sold as tablets or in powder blends, as follows:

Activity for powder for tablets	>1600 BMK/g
Activity for powder for powder blends	1000 - 1500 BMK/g
Dry Matter	>91.0%

Dry Matter is measured by determining the loss on drying after 4 hours at 105°C and expressing as percentage change in weight. The tableted products also contain microcrystalline cellulose, sorbitol, wheat starch and sodium chloride while the powders contain microcrystalline cellulose, wheat starch and sodium chloride or microcrystalline cellulose, calcium carbonate, citric acid and BHT, depending upon the product.

5.2 Specifications

The G4-amylase preparation conforms to the general and additional requirements for enzyme preparations as described in the FCC[23]. In addition, the G4-amylase preparation also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by JEFCA[24].

The following specifications have been established for the G4-amylase and apply to both the liquid and dry products:

(See next page)

GENENCOR

Property	Method Number	Reference Method	Specification
ENZYME ACTIVITIES			
Amylase, betamyl units	Danisco/Genencor Method	150,000 BMU/g (liquid) 1000 - 1500 BMK/g (powder for blends) >1600BMK/g (tablets)	Amylase, betamyl units
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	800V	ISO 4833 - "Microbiology -General guidance for the enumeration of micro-organisms - colony count technique at 30°C" and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Not more than 50,000 CFU/g
Total Coliforms	810V	ISO 4832 - "General guidance for the enumeration of coliforms - colony count technique" and the FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Not more than 30 CFU/g
E. coli	819V	ISO 7251 - Microbiology - "General Guidance for Enumeration of Presumptive *Escherichia coli* - Most Probable Number Technique" and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Negative/25g
Salmonella	832V	Nordic Committee on Food Analysis; Salmonella Bacteria; Detection in Foods. No 71; 4th Edition; 1991 and FDA	Negative/25g

Property	Method Number	Reference Method	Specification
		Bacteriological Analytical Manual; 8th Edition; AOAC International	
Production strain	892V	Genencor Method	Negative by test
Antibacterial Activity	899V	FAO Food and Nutrition Paper: 25th Session of the Joint FAO/WHO Expert Committee on Food Additives; Geneva 1981; p317-318; Appendix A	Negative by test
OTHER ASSAYS			
Lead	603W-PB	AAS/ICP-AES method in Jecfa, Combined Compendium of Food Additive Specifications, Volume 4, Rome, 2006	Less than 5 mg/kg

Lead and antibacterial activity are analyzed at regular intervals. Activity and microbial specifications are analyzed on every production batch of enzyme.

The lead, Coliforms and Salmonella specifications meet FCC and JEFCA requirements. The *E. coli* and antibacterial activity specifications meet JEFCA requirements and are not included in FCC. The production microorganism specification is a Danisco specification and is not mentioned in FCC or JEFCA.

The G4-amylase assay is colorimetric and monitors the rate of degradation of p-nitrophenyl maltoheptoside. Alpha-glucosidase and a glucoamylase are used as coupling enzymes. This substrate has the non-reducing terminal sugar chemically blocked, thus preventing any interaction with glucoamylase. The rate of p-nitrophenyl release is proportional to amylase activity and is monitored at 410nm. Note that this procedure involves a stop reaction at alkaline pH. The alkaline pH is necessary to stop the reaction and to provide optimal color development for the endpoint reaction.

The activity of Amylase SAS3 is defined in BMU units (liquid) and BMK units (dry). One BMK unit equals 1000 BMUs. This unit is not defined in exact terms, but relies on a specific assay and a standard enzyme. The assay is based on the enzyme's ability to break down blocked p-nitrophenyl maltoheptaoside. The specific activity of Amylase SAS3 is 3.0 BMU/mg enzyme protein using this assay.

6. APPLICATION

6.1 Mode of Action

There are two main applications of amylases in baking. The first is flour standardization, *i.e.* optimization of α-amylase activity in the flour, and the second is anti-staling.

Fungal α-amylase (EC 3.2.1.1) from *Aspergillus oryzae* is added to flour to optimise α-amylase activity. Use of fungal α-amylase in bread baking was approved in 1955 in the US and fungal α-amylase has since become the most widely used enzyme in baking world wide. Danisco started the marketing of fungal enzymes containing fungal α-amylase and xylanase activities to the milling and baking industry back in the 1980s.

The second important objective for use of amylases in baking is anti-staling, i.e., extending the freshness of baked goods. Staling is a highly complex phenomenon with firming being the most well-known and important symptom. Anti-staling amylases have to modify starch during the baking process to provide significant anti-staling effects. As intact starch granules are hardly attacked anti-staling amylases have to be sufficiently heat-stable to be active during the baking step after initial gelatinization of starch granules.

Endo-amylases (EC 3.2.1.1) are used for anti-staling and work by degrading amylose so that a weaker amylose network is formed during starch retrogradation after baking. Exo-amylases such as maltogenic amylase (EC 3.2.1.133) cloned from *Bacillus stearothermophilus* and now maltotetraohydrolase (EC 3.2.1.60) cloned from *P. saccharophila* offer clear improvements for anti-staling applications. By shortening the amylopectin side chains and releasing malto-oligosaccharides, they efficiently reduce staling by lowering the rate of amylopectin retrogradation without disadvantageous side effects caused by excessive weakening of the amylose network.

To our knowledge, maltotetraohydrolase (EC 3.2.1.60) from *P. saccharophila/stutzeri* has only been used in baking via two Danisco products which were pre-cursors to this G4-amylase. Closely related maltotetraohydrolases (EC 3.2.1.60) from *P. stutzeri* strains have been used for producing maltotetraose and maltotetraose syrups marketed for use in foods since the 1980s[25]. Hayashibara Company Ltd markets a maltotetraose syrup called Tetrup which is produced using maltotetraohydrolase (http://www.hayashibara.co.jp/eng/contents_pdf.html) and holds patent EP289138B1 on maltotetraose-producing amylase from e.g. *P. stutzeri* and its use for maltotetraose production.

Maltotetraohydrolase (EC 3.2.1.60) belongs, like fungal α-amylase from *A. oryzae* (EC 3.2.1.1) and maltogenic amylase (EC 3.2.1.133), to the so-called α-amylase family or glycoside hydrolase family 13. It is a large enzyme family constituting many different reactions and product specificities (http://afmb.cnrs-mrs.fr/CAZY/) and sharing a common $(ß/\alpha)_8$- or TIM-barrel catalytic domain. It consists of a closed eight-stranded parallel ß-sheet surrounded by eight α-helices. Four short conserved sequence motifs with seven residues strictly conserved in the α-amylase family are involved in forming the active site at the C-terminal ends of the ß-strands in

the $(\beta/\alpha)_8$-barrel. These four conserved sequence regions of the α-amylase family enzymes contain the three conserved catalytic residues Asp, Glu and Asp.

CAZy Family	Glycoside Hydrolase Family 13
Known Activities	α-amylase (EC 3.2.1.1); pullulanase (EC 3.2.1.41); cyclomaltodextrin glucanotransferase (EC 2.4.1.19); cyclomaltodextrinase (EC 3.2.1.54); trehalose-6-phosphate hydrolase (EC 3.2.1.93); oligo-α-glucosidase (EC 3.2.1.10); maltogenic amylase (EC 3.2.1.133); neopullulanase (EC 3.2.1.135); α-glucosidase (EC 3.2.1.20); maltotetraose-forming α-amylase (EC 3.2.1.60); isoamylase (EC 3.2.1.68); glucodextranase (EC 3.2.1.70); maltohexaose-forming α-amylase (EC 3.2.1.98); branching enzyme (EC 2.4.1.18); trehalose synthase (EC 5.4.99.16); 4-α-glucanotransferase (EC 2.4.1.25); maltopentaose-forming α-amylase (EC 3.2.1.-) ; amylosucrase (EC 2.4.1.4) ; sucrose phosphorylase (EC 2.4.1.7); malto-oligosyltrehalose trehalohydrolase (EC 2.4.1.141); isomaltulose synthase (EC 5.4.99.11).
Mechanism	Retaining
Catalytic Nucleophile/Base	Asp (experimental)
Catalytic Proton Donor	Glu (experimental)
3D Structure Status	Available (see PDB). Fold $(\beta/\alpha)_8$

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The enzyme preparation will be used in bakery products such as bread, bread buns, tortillas and crackers to delay the staling of these bakery products.

6.2 Use Levels

To obtain the desired anti-staling effects of this G4-amylase, the recommended dose is 2.0-30.0 mg enzyme protein/kg flour (2.28 – 34.2 mg TOS/kg flour).

6.3 Enzyme Residues in the Final Foods

The enzyme is largely inactivated during baking and has no further technical effect after baking

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

The safety of the production organism must be the prime consideration in assessing the safety of an enzyme preparation intended for use in food. [3] If the organism is non-toxigenic and non-pathogenic, then it is assumed that foods or food ingredients produced from the organism, using current Good Manufacturing Practices, are safe to consume.[4] Pariza and Foster[3] define a non-toxigenic organism as 'one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure' and a non-pathogenic organism as 'one that is very unlikely to produce disease under ordinary circumstances.' *B. licheniformis* meets these criteria for non-toxigenicity and non-pathogenicity.

7.1.1 Safety of the host

Bacillus licheniformis is considered a Class 1 Containment Agent under the NIH Guidelines for Research Involving Recombinant DNA Molecules and by the Ministerie van de Vlaamse Gemeenschap in Flanders, Belgium, and is also considered as GILSP worldwide.

Mixed carbohydrase and protease from *B. licheniformis* were affirmed as GRAS by FDA on January 4, 1983 (48 FR 239). Also the FDA has no questions to four GRAS notices on enzymes derived from *B. licheniformis*:

- Alpha-amylase derived from *B. licheniformis* carrying a gene encoding a modified alpha-amylase derived from *B. licheniformis* and *Bacillus amyloliquefaciens* (GRN No. 22),
- Alpha-Amylase derived from *Bacillus licheniformis* carrying a gene encoding alpha-amylase from *B. stearothermophilus* (GRN No. 24),
- Pullulanase derived from *B. licheniformis* carrying a gene encoding pullulanase from *Bacillus deramificans*(GRN No. 72), and
- Alpha-amylase derived from *B. licheniformis* carrying a gene encoding a modified alpha-amylase from *B. licheniformis* (GRN No. 79).

Amylase from *B. licheniformis* has been reviewed by the Joint Expert Committee on Food Additives (JECFA) of FAO/WHO and an acceptable daily intake (ADI) "not specified" has been set[26].

B. licheniformis, including genetically modified strains, has been approved for the production of amylase enzymes in the food industry in France and it is also approved for the production of proteases and pullulanase (Arrêté du 05/09/1989 et compléments).

Strains of *B. licheniformis* are found in Table V of Division 16 of "Canadian Food and Drugs Act and Regulations", as an authorized source for amylases and proteases in several food applications.

The species *B. licheniformis* is an accepted source of safe food enzymes in the literature. The safety of *B. licheniformis* strains was recently reviewed by Priest *et al*[27]. It is also recognized as a safe host strain for recombinantly derived enzymes by Olempska-Beer *et al*[28].

Pathogenic strains of *B. licheniformis* are NOT described in the Bergey Manual[10] or in the ATCC and other catalogues. The species *B. licheniformis* does not appear on the Proposal for a Council Directive amending the "Directive 90/679/EEC on the protection of workers from risks related to exposure to biological agents at work".

Strains of *B. licheniformis* are found in the Sixth edition of "Food Chemicals Codex" as a source for the enzymatic preparation of carbohydrase and protease used in the treatment of foods.

The parent strain of the current production strain and its progeny, *B. licheniformis* BRA7, have been used by Genencor for the production of α-amylase enzyme preparations since 1989, as well as for the production of proteases, pullulanase and xylanase.

Numerous oral toxicity mutagenicity and carcinogenicity studies using enzyme products from *B. licheniformis* BRA7-derived strains have been performed, and no evidence of a toxic or mutagenic effect has been observed.

7.1.2 Safety of the donor source

See section 3.3.

7.2 Safety of the Manufacturing Process

The manufacturing process for the production of G4-amylase will be conducted in a manner similar to other food and feed production processes. It consists of a pure-culture fermentation process, cell separation, concentration, filtration and formulation, resulting in a liquid G4-amylase enzyme concentrate that is then dried and agglomerated for use in baking. The process, described in Section 4, is conducted in accordance with FDA's cGMPs as set forth in 21 C.F.R. Part 110. The resultant products meet the general requirements for enzyme preparations of the FCC[23] and WHO/JEFCA[24].

7.3 Safety of G4-amylase

7.3.1 Allergenicity

According to Pariza and Foster[3], there have been no confirmed reports of allergies in consumers caused by enzymes used in food processing. Amylases have been used extensively in food processes for over 50 years and have generated no known safety concerns. It is well-known that occupational exposure to airborne flour dust and enzyme particles can lead to dermal and respiratory sensitization among industry workers (AMFEP, 1998). The rate of such occurrences is relatively low, however. Studies have shown that occupational exposure does not give rise to allergic responses among workers or consumers upon ingestion of enzyme-treated baked goods. For example, Cullinan *et al*[30] studied 17 adults with demonstrated sensitivity to *Aspergillus* species (positive skin prick response). Subjects were fed bread containing *Aspergillus*-derived α-amylase or hemicellulase and an enzyme-free control bread on consecutive days. There were no consistent clinical or functional reactions to active challenge in any of the subjects.

In 1998 the Association of Manufacturers of Fermentation Enzyme Products (AMFEP) Working Group on Consumer Allergy Risk from Enzyme Residues in Food reported on an in-depth analysis of the allergenicity of enzyme products. They concluded that there are no scientific indications that small amounts of enzymes in bread and other foods can sensitize or induce allergy reactions in consumers, and that enzyme residues in bread and other foods do not represent any unacceptable risk to consumers. Further, in a recent investigation of possible oral allergencity of 19 commercial enzymes used in the food industry, there were no findings of clinical relevance even in individuals with inhalation allergies to the same enzymes, and the authors concluded "that ingestion of food enzymes in general is not considered to be a concern with regard to food allergy[31]."

Despite this lack of general concern, the potential that G-4 amylase could be a food allergen was examined. FASTA alignments of the full SAS3 sequence with known allergens using either the SDAP database (http://fermi.utmb.edu/SDAP/sdap_who.html)containing 737 allergens or the Allermatch

database combined database (www.allermatch.org) containing 792 allergens suggests a low degree of homology (approx. 20%, E score 10^{-8}) with Asp o 21 (*Aspergillus oryzae* TAKA amylase a, sequences AAA32708 and BAA00336 in Genbank and described in Tsukagoshi et al.[34]). The low degree of homology in itself does not imply SAS3 to be a food allergen.

Following the recommendations in FAO/WHO[35], two additional types of searches against the combined Allermatch database containing non-redundant allergen sequences from the SwissProt database and the WHO-IUIS list were performed using Allermatch software (Fiers et al., [36]) as well as against the SDAP database were performed.

The initial search according to the FAO/WHO guidelines, using a sliding window of 80-amino acid stretches and greater than 35% identity to indicate a match, revealed no matches of SAS3 with known allergens. A secondary, more detailed search for exact matches of short stretches (6 amino acids) of sequence that could serve as potential IgE binding sites indicated that no such short matching stretches exist, including to the *A. orzyae* amylase, confirming that SAS3 is not a food allergen.

Using the FAO/WHO recommendations, we conclude that SAS3 is unlikely to be a food allergen.

7.3.2 Safety Studies

7.3.2.1 Test item

The UF Concentrate was prepared as the test item for the safety studies. UF concentrate is the ultrafiltered concentrate of the enzyme preparation, stopped during the manufacturing process before the addition of formulation ingredients. The UF concentrate is characterized by:

Enzyme activity	241318 BMU/g or 3000 BMU/mg enzyme protein
pH	4.93
Specific gravity	1.028
%TOS (100 –%ash-%moisture)	9.09%
Total Protein content	78.76 mg/ml

The UF concentrate was stored frozen in aliquots and thawed before use.

The test item was used to conduct the following studies:

Study
Acute dermal irritation study in rabbits (sequential approach)
Acute Eye Irritation/Corrosion Study in the Rabbit
Acute oral toxicity in rats – Fixed dose procedure
Sub-chronic 13 week toxicity in the rat

Bacterial Reverse Mutation Assay – Ames assay
In vitro chromosomal aberration Study.

7.3.2.2 Acute dermal irritation study in rabbits (sequential approach)

A. Procedure:

The objective of this study was to assess the local irritant effect of this G4-amylase. This study was conducted according to the method recommended in the OECD Guideline No. 404 "Acute dermal irritation/corrosion" (adopted 24 April 2002) and was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999) and the requirements of Directives 2004/9/EC and 2004/10/EC.

In the initial test, the back of one rabbit was shaved and three test sites were selected. At each test site a quantity of 0.5 ml of the test material was placed under a cotton gauze patch, which was secured in position with a strip of adhesive tape. One patch was removed at each of three time points: 3 minutes, 1 hour and 4 hours after application.

In the confirmatory test, two additional rabbits were treated with 0.5 ml of the test material. One patch was applied and was allowed to remain in contact for 4 hours. The test sites were examined for irritation approximately one hour following the removal of the patches and at 24, 48 and 72 hours later.

B. Results

No deaths or overt signs of toxicity were observed in this study. No effects on feed consumption and weight gain were recorded. No erythema, eschar or edema was observed at these test sites at any of the examinations throughout the study.

The test material produced a primary irritation index of 0.0 and no corrosive effects were noted.

C. Evaluation

On the basis of the results obtained in this study, this G4-amylase is classified as non-irritant according to OECD Guideline No 404, "Acute Dermal Irritation/Corrosion", April 2002, and the Official Journal of the European Communities, L 110 A, volume 36, 4 May 1993 § 3.2.5 ("C", R35 or R34) and § 3.2.6.1 ("Xi", R38).

7.3.2.3 Acute Eye Irritation/Corrosion Study in the Rabbit

A. Procedure

The objective of this study was to assess the ocular irritation potential of this G4-amylase. This study was conducted according to the method recommended in the OECD Guideline No. 405 "Acute eye irritation/corrosion" (adopted 24 April 2002) and was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999) and the requirements of Directives 2004/9/EC and 2004/10/EC.

In the initial test, the test material was applied at 0.1 ml to the conjunctival sac of the right eye. The left eye served as control. Assessment of ocular damage/irritation was made at approximately 1 hour and at 24, 48 and 72 hours after application. Examination of the eye was facilitated by the use of a light source from a standard ophthalmoscope. In the confirmatory test, two additional rabbits were used.

B. Results

One rabbit was killed for humane reasons, immediately after the 48-hour observation period, due to a broken front limb. As full recovery from ocular effects had already been noted by the time of its sacrifice, the absence of this rabbit was considered not to affect the purpose or integrity of the study.

No corneal or iridial effects were noted. Minimal to moderate conjunctival irritation was noted in all treated eyes one hour after treatment. At the 24-hour observation period, only minimal conjunctival irritation was noted. All eyes were cleared by the 48 and 72-hour periods. The test material produced a maximum group mean score (PIS = primary irritation score) of 7.3/110.0 at the 1 hour observation period, a PIS of 0.7/110.0 at the 48-hour observation period and a PIS of 0.0/110.0 at the 72-hour observation period.

C. Evaluation

The maximal primary eye irritation score was 7.3/110.0. According to the EEC Directive published in: EEC Directive published in: "Official Journal of the European Communities" No: L 383 A, volume 35, 29.12.1992, part B5: Acute toxicity (eye irritation) and No: L 110 A, volume 36, 04.05.1993, part 3.2.6.2 Ocular lesions (which is implemented in Commission Directive 2001/59/EC of 6 August 2001), this G4-amylase is classified as mild irritant.

7.3.2.4 Acute oral toxicity in rats – Fixed dose procedure

A. Procedure:

The objective of this study was to assess the acute toxicity of this G4-amylase when administered as a single oral dose followed by a 14-day period of observation. The information is used for both hazard assessment and ranking purposes. The study was initiated with a sighting study using 2000 mg/kg with one female Sprague Dawley CD rat. In the absence of toxicity at a dose level of 2000 mg/kg, an additional group of 4 female rats was dosed with 2000 mg/kg bw.

This study was conducted according to OECD Guideline No. 420 "Acute oral toxicity – Fixed dose method" (adopted 17 December 2001) and was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999) and the requirements of Directives 2004/9/EC and 2004/10/EC.

B. Results:

No mortality was recorded in this study. There were no overt signs of systemic toxicity throughout the 14-day observation period and at necropsy.

C. Evaluation:

Under the conditions of this study, the oral LD_{50} was equal or greater than 2000 mg/kg bw corresponding to GHS category 4 according to Annex 3 of the OECD Guideline for Testing of Chemicals No. 420. This G4-amylase is classified as "Practically non toxic by ingestion".

7.3.2.5 A 13-week Oral (Gavage) Toxicity Study in Rats

A. Procedure

The objective of this study was to investigate the potential of this G4-amylase to induce systemic toxicity after repeated daily oral administration to Wistar rats of both sexes for 90 consecutive days. Groups of 10 rats/sex each were gavaged daily with 0 (0.9% saline), 0, 23.7, 47.4 or 79 mg total protein/kg body weight in a constant volume of 5 ml 0.9% saline/kg body weight corresponding to 0, 27.3, 54.5 or 90.9 mg TOS/kg bw/day, respectively.

Animals of the same sex were housed three or four in solid floor polypropylene cages with softwood flake bedding (Harlan UK, Oxon) and had access to water (via bottle) and feed *ad libitum*. A pelleted diet (Rodent 2014C, Harlan UK, Oxon) was used. For environmental enrichment, the animals were provided a

supply of Aspen Wood Wool at each change of bedding. All groups were housed under controlled temperature, humidity and lightning conditions.

All animals were observed daily for mortality and signs of morbidity. Body weight and feed consumption were recorded weekly. Water consumption was recorded twice weekly for each cage. Ophthalmologic examination was performed on all animals prior to study initiation and in the control and high dose groups at study termination. Hematology was conducted on Day 0, 30 and 90. A functional observation battery consisting of detailed clinical observation, reactivity to handling and stimuli and motor activity examination was conducted during week 13 for all animals. Clinical chemistry was evaluated at study termination prior to necropsy on all groups. After a thorough macroscopic examination, selected organs were removed, weighed and processed for future histopathologic examination. Microscopic examination was conducted on selected organs from control and high dose animals. If a questionable finding was noted, the microscopic examination would be extended to the low and mid dose groups.

This study was conducted in accordance with OECD guideline No. 408 "Subchronic oral toxicity – Rodent: 90 day study" (adopted 21 September 1998), EPA Guideline OPPTS 870.3100 (August 1998) and was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999) and the requirements of Directives 2004/9/EC and 2004/10/EC.

B. Results

No treatment-related deaths were recorded throughout the entire investigation. One low dose female was killed *in extremis* on day 68 following signs of lethargy, pilo-erection, hunched posture and staining around the snout. These observations were considered as non-treatment related. One mid dose female was found dead on day 90 just prior to terminal kill. This animal did not show any clinical signs prior to death.

Weekly open-field arena observations did not reveal any treatment-related effects. No treatment-related changes in functional performance tests were detected. No treatment-related changes in sensory reactivities were detected in the treated groups as compared with controls. There were no biological or statistical differences between the control and treated groups with respect to body weights, feed consumption, water consumption, feed efficiency (ratio of body weight gain to dietary intake), hematology, and ophthalmologic examinations.

Females from all treated groups showed an increase in serum potassium in comparison to concurrent controls. On review of historical control data, these increases were attributed to lower than expected concurrent control values. The

increases in serum potassium were not statistically different from historical control values.

Females from all treated groups showed a decrease in absolute and relative ovary weights when compared to concurrent controls. On review of historical control data, the control values for this parameter were higher than the expected ranges for female rats of the age and strain employed. In the absence of histopathologic findings, these findings were not attributed to treatment. Incidental macroscopic findings were noted (one high dose male displayed small kidneys; one high dose male with hydronephrosis on the right kidney) but were not considered as treatment-related in the absence of histopathologic changes. Scattered histopathologic findings were noted, but all morphological changes were those commonly observed in rats of the age and strain employed and there were no differences in severity or incidence between control and treated groups.

C. Evaluation

There were 2 interim deaths in this study but both were considered as non-treatment related. Blood chemical changes were confined to females and consisted of an increase in serum potassium. Since the changes were within the range of the historical control data, they were not considered as treatment-related. Significant decreases in absolute and relative ovary weights were noted when compared to concurrent control values. However, on review of historical control data, the control values for this parameter were higher than the expected ranges for female rats of the age and strain employed. Therefore, these findings were considered as non-treatment related especially in the absence of histopathological changes in the ovaries.

It can be concluded that daily administration of G4-amylase by gavage for 90 consecutive days did not result in adverse systemic toxicity or adverse effects on clinical chemistry, hematology, functional observation tests and macroscopic and histopathologic examinations. Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established at the highest dose tested, 79 mg total protein/kg bw/day corresponding to 90.0 mg TOS/kg bw/day and 241318 BMU/kg bw/day.

7.3.2.6 Bacterial Reverse Mutation Assay – Ames assay

A. Procedure:

The objective of this assay was to assess the potential of G4-amylase to induce point mutations (frame-shift and base-pair) in four strains of *Salmonella typhimurium* TA 98, TA 100, TA 1535 and TA 1537 and *E. coli* strain WP2uvrA$^-$. The test material was tested both in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). The tests were

performed using the plate incorporation method, at 5 dose levels, in triplicate. The dose levels selected for the assay were based on the total protein content of the test material (1 ml contained 79 mg total protein).

A preliminary toxicity test was carried out with dose levels ranging from 0.15 to 5000 µg/plate.

The test material (0.1 ml) was mixed with 0.1 ml of bacterial suspension, 2 ml of molten agar, 0.5 ml of S-9 mix or phosphate buffer and overlaying onto sterile plates of agar. Sterile distilled water served as vehicle control. After 48 hours incubation at 37°C, the plates were assessed for the number of revertant colonies and growth of the bacterial background lawn.

In the main assay, five concentrations of the test material were assayed in triplicate against each tester strain in both the presence and absence of metabolic activation. The test material (0.1 ml) was mixed with 0.1 ml of bacterial suspension, 2 ml of molten agar, 0.5 ml of S-9 mix or phosphate buffer and overlaying onto sterile plates of agar. Sterile distilled water served as vehicle control. The positive controls used for plates without S-9 mix were N-ethyl-N'-nitro-nitrosoguanidine, 9-aminoacridine and 4-nitroquinoline-1-oxide. The positive controls used for plates with S-9 mix were 2-aminoanthracene and benzo(a)pyrene. After 48 hours incubation at 37°C, the plates were assessed for the number of revertant colonies and growth of the bacterial background lawn.

All dose levels were expressed in terms of total protein. The highest dose level tested (5000 µg/plate) is the maximum required by the OECD guideline. This assay was conducted in accordance with OECD guideline No. 471 "Bacterial reverse mutation test", the US EPA (TSCA) OPPTS harmonized guidelines, mutagenicity guidelines published by the Japanese Regulatory Authorities (METI, MHLW and MAFF) and was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999) and the requirements of Directives 2004/9/EC and 2004/10/EC.

B. Results:

In the screening assay, G4-amylase was not toxic to the test bacteria up to and including the highest dose level tested (5000 µg/plate) in both the presence and absence of S-9 mix. The test material caused no visible reduction in the growth of the bacterial background lawn at any dose level. Therefore, 5000 µg/plate was selected as the highest dose level for the main tests.

In the main tests, five dose levels (50, 150, 500, 1500 and 5000 µg/plate) were used. Insoluble material was observed at and above the 1500 µg/plate but this did not prevent the scoring of the revertant colonies. No biologically or statistically significant increases in the number of revertant colonies were observed in any

dose level in either main test. Statistical increases in the number of revertant colonies were noted with the positive controls in both the presence and absence of metabolic activation substantiating the sensitivity of the treat and plate assay and the efficacy of the metabolic activation mixture.

C. Evaluation

Under the conditions of this assay, G4-amylase has not shown any evidence of mutagenic activity in the Ames assay.

7.3.2.7 *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes

A. Procedure

The objective of this assay was to investigate the potential of G4-amylase to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). G4-amylase was mixed with cultures of human peripheral lymphocytes both in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver; S-9 mix). This assay consisted of a preliminary toxicity (dose range finding) assay and two main tests. Five concentrations of G4-amylase were used in the preliminary assay and at least 3 dose levels were then selected for the two main assays with the highest dose level clearly inducing a toxic effect (50% reduction in mitotic index). In the absence of cytotoxicity, the highest dose selected would be 5000 µg/ml, as recommended by the OECD guideline.

With metabolic activation:
Cells were grown in Eagle's essential medium with HEPES buffer (MEM) at 37°C with 5%CO2 in air. After 48 hours, the cell cultures were transferred to tubes and centrifuged. Approximately 9 ml of the culture medium was removed and G4-amylase (1 ml), S-9 mix (1ml), and MEM culture media (7ml) were added to the cell cultures and incubated at 37°C for 4 hours. The cultures were then centrifuged, the treatment medium removed and washed with MEM culture medium. After a further centrifugation, the MEM culture medium was removed by suction and replaced with the fresh MEM culture medium. The cell cultures were then re-incubated for an additional 20 hours at 37°C in 5% CO_2 in humidified air.

Without metabolic activation:
Cells were grown in Eagle's essential medium with HEPES buffer (MEM) at 37°C with 5%CO2 in air. After 48 hours, the cell cultures were transferred to tubes and centrifuged. Approximately 9 ml of the culture medium was removed and G4-amylase (1 ml) and MEM culture media

(8ml) were added to the cell cultures and incubated at 37°C for 4 hours. The cultures were then centrifuged, the treatment medium removed and washed with MEM culture medium. After a further centrifugation, the MEM culture medium was removed by suction and replaced with the fresh MEM culture medium. The cell cultures were then re-incubated for an additional 20 hours at 37°C in 5% CO_2 in humidified air.

All cultures (with and without S-9 mix) were harvested 24 hours after the start of treatment. Two hours prior to harvest, Demecolcine (0.1 μg/ml) was added to all cultures to arrest all cells at the metaphase-stage of mitosis. At the harvest time, all cultures were centrifuged and the supernatant discarded. The cell pellets were resuspended in a KCl solution, incubated for 10 minutes, centrifuged and the supernatant removed. The cells were then fixed on slides, stained and scored for chromosomal aberrations:

a. Cytotoxicity was evaluated using the mitotic index (number of cells in mitosis/1000 cells examined. From these results, a dose level causing a decrease in mitotic index of 50% was selected as the highest dose in the main tests.

b. Metaphase analysis (i.e. evaluation of chromosomal aberration) was conducted on 100 metaphases per dose level.

c. Mitomycin C and cyclophosphamide were used as positive controls for cultures without S-9 mix and with S-9 mix, respectively.

This assay was conducted in accordance with OECD guideline No. 473 "*In vitro* Mammalian chromosome aberration test" and was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999) and the requirements of Directives 2004/9/EC and 2004/10/EC.

B. Results

In the preliminary assay, the dose range was 19.53 to 5000 μg/ml. Cytotoxicity (only 6% survival) was noted at the 312.5 μg/ml dose level. Therefore, the maximum dose level selected for the 4-hour incubation period in the presence and absence of S-9 mix was 625 μg/ml and the maximum dose selected for the 24-hour continuous exposure was 312.5 μg/ml.

In both main assays, G4-amylase did not induce a statistically significant increase in the frequency of cells with aberrations either in the absence or presence of metabolic activation.

In both main assays, G4-amylase did not induce a statistically significant increase in the numbers of polyploid cells at any dose level either in the presence or absence of metabolic activation.

In both main assays, significant increases in aberrant metaphases were noted with the positive controls demonstrating the sensitivity of the tests and the efficacy of the S-9 mix.

C. Evaluation

Under the conditions of this test, G4-amylase did not induce chromosomal aberrations (both structural and numerical) in this *in* vitro cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation.

7. 4. Overall Safety Assessment and Human Exposure

According to the Directive of the Commission 93/21/EEC of April 27, 1993, G4-amylase is non hazardous based on acute oral and irritation studies. In genotoxicity studies, G4-amylase is not mutagenic, clastogenic or aneugenic. Daily oral administration of G4-amylase up to and including a dose level of 79 mg total protein/kg bw/day (90.0 mg TOS/kg bw/day) does not result in any manifestation of systemic, hematologic, functional or histopathologic adverse effects.

7.4.1 Identification of the NOAEL

In the 90-day oral (gavage) study in rats (SafePharm Lab No. 2420-0008), a NOAEL was established at 79 mg total protein/kg bw/day (equivalent to 90.0 mg TOS/kg bw/day). The study was conducted in compliance with both the UK and OECD Good Laboratory Practice Regulations and was designed based on OECD guideline No. 408. Since human exposure to G4-amylase is through oral ingestion, selection of this NOAEL is thus appropriate.

No Observed Adverse Effect Level = 79 mg total protein/kg bw/day
= 90.0 mg TOS/kg bw/day

7.4.2 Human Exposure Assessment

G4-amylase is used in bakery. Based on the Food Consumption Statistics collected from 1979-1988 by OECD (1991), Italy has the highest daily consumption of bread, i.e. 4.92 g/kg bw/day. Data from the USDA, Agricultural Research Service (1997) reveals the

maximum daily bread consumption in the US as 4.07 g/kg bw/day. Therefore, for the purpose of this safety assessment:

1. The maximum daily bread consumption of 4.92 g/kg bw/day is used to represent a worst-case scenario:
   Maximum daily bread consumption = 4.92 g/kg bw/day;
2. Since the flour content in bread is 66%, the maximum daily flour consumption is:
   4.92 g/kg bw/d X 66% = 3.25 g/kg bw/day;
3. G4-amylase is applied at the recommended range of 2.0 to 30.0 mg enzyme protein/kg flour;
4. Maximum intake of G4-amylase from flour:
   3.25 g/kg bw/day X 30 mg/kg flour = 0.098 mg enzyme protein/kg bw/day;
5. Daily exposure to G4-amylase (worst case) assuming that 100% of enzyme remains in the bread, not removed or inactivated by heat and also on the assumption of 100% market penetration =
   0.098 mg enzyme protein/kg bw/day (0.11 mg TOS/kg bw/day)

Risk Assessment

Risk for humans through ingestion of bakery made from flour treated with G4-amylase is measured by a Margin of Exposure (MOE), which determines how close the estimated daily human exposure comes to a No Observable Adverse Effect Level (NOAEL). Generally, a MOE greater than 100 means consumption is not of concern.

$$\text{Margin of exposure} = \frac{\text{No observed adverse effect level}}{\text{Daily exposure}}$$

Based on the results from the 90-day oral (gavage) feeding study cited above,

$$\text{Margin of exposure} = \frac{\text{79 mg enzyme protein/kg bw/day}}{\text{0.098 mg/kg bw/day}} = 806.$$

7.5 Conclusion

The safety of G4-amylase is assessed in a battery of toxicology studies investigating its acute oral, irritation, mutagenic and systemic toxicity potential. G4-amylase is not a skin irritant, is not acutely toxic by ingestion and is a mild eye irritant. A battery of genotoxicity assays was conducted and under the conditions of these assays G4-amylase is not a mutagen, a clastogen, or an aneugen.

Daily administration of G4-amylase by gavage for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at 79 mg total protein/kg bw/day (equivalent to 90.0 mg TOS/kg bw/day).

Even under the worst case scenario that G4-amylase is applied at the maximum rate (30 mg enzyme protein/kg flour) and the enzyme is not destroyed and/or removed during baking, the use

of G4-amylase as a food additive in bakery is not expected to result in adverse effects to humans. Based on the NOAEL of 79.0 mg total protein/kg bw/day (90.0 mg TOS/kg bw/day), a safety margin of 806-fold exists for the proposed uses.

8. BASIS FOR GENERAL RECOGNITION OF SAFETY

As noted in the Safety sections above, enzyme preparations derived from *B. licheniformis*, including α-amylase, protease, pullulanase, and xylanase, are well recognized by qualified experts as being safe. Published literature, government laws and regulations, reviews by expert panels such as FAO/WHO JECFA, as well as Genencor's own unpublished safety studies, support such a conclusion.

Bacillus licheniformis is widely used by enzyme manufacturers around the world for the production of enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. It is a known safe host for enzyme production. An enzyme preparation derived from a recombinant microorganism will be safe if the host microorganism is nonpathogenic and non-toxigenic; the genetic information that is introduced into the host microorganism is well characterized; and the added DNA does not encode and express any known harmful or toxic substances. Information is provided in this document to support that the components of the enzyme preparation meet these standards.

The safety studies conducted by Genencor established a NOAEL of 79.0 mg total protein/kg bw/day (90.0 mg TOS/kg bw/day) and a safety margin of 806-fold for the proposed uses.

Utilizing the information provided, the enzyme preparation was taken through the Pariza-Johnson decision tree for microbial enzymes (see Appendix 3) and the preparation was determined to be acceptable for use in foods as described above.

Based on the available data from the literature and generated by Genencor, the company has concluded that the enzyme preparation from *B. licheniformis* strain GICC03279 is safe and suitable for use in bakery products.

In addition, the safety determination including construction of the production organism, the production process and materials, and safety of the product were reviewed by Dr. Michael W. Pariza, who concurred with the company's conclusion that the product is GRAS (see Appendix 4) (note, the safety studies on this G4-amylase were conducted after the GRAS determination by Dr. Pariza to support regulatory approvals outside the US where toxicology testing is required).

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10. LIST OF APPENDICES

Appendix 1 – The amino acid sequence of the G4-amylase

Appendix 2 - The Manufacturing process

Appendix 3 – The Pariza/Johnson Decision Tree

Appendix 4 – GRAS Panel Report

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Appendix 1 – The amino acid sequence of this G4-amylase

Amino acid sequence alignment of this G4-amylase vs. PS4 wild type Maltotetraohydrolase. The amino acids residue positions changed between wild type PS4 and this G4-amylase are shown in green.

```
PS4 MATURE      (     1) -dqagkspagvryhggdeiilqgfhwnvvreapndwynilrqqastiaad
This G4 MATURE  (     1) ...................................................

PS4 MATURE      (    50) gfsaiwmpvpwrdfsswtdggksgggegyfwhdfnkngrygsdaqlrqaa
This G4 MATURE  (    51) ...................................................

PS4 MATURE      (   100) galggagvkvlydvvpnhmnrgypdkeinlpagqgfwrndcadpgnypnd
This G4 MATURE  (   101) ...................................................

PS4 MATURE      (   150) cddgdrfiggesdlntghpqiygmfrdelanlrsgygaggfrfdfvrgya
This G4 MATURE  (   151) ...................................................

PS4 MATURE      (   200) pervdswmsdsadssfcvgelwkgpseypswdwrntaswqqiikdwsdra
This G4 MATURE  (   201) ...................................................

PS4 MATURE      (   250) kcpvfdfalkermqngsvadwkhglngnpdprwrevavtfvdnhdtgysp
This G4 MATURE  (   251) ...................................................

PS4 MATURE      (   300) gqnggqhhwalqdglirqayayiltspgtpvvywshmydwgygdfirqli
This G4 MATURE  (   301) ...................................................

PS4 MATURE      (   350) qvrrtagvradsaisfhsgysglvatvsgsqqtlvvalnsdlanpgqvas
This G4 MATURE  (   351) ...................................................

PS4 MATURE      (   400) gsfseavnasngqvrvwrsgsgdgggndggegglvnvnfrcdngvtqmgd
This G4 MATURE  (   401) ---------------------------------------------------

PS4 MATURE      (   450) svyavgnvsqlgnwspasavrltdtssyptwkgsialpdgqnvewkclir
This G4 MATURE  (   450) ---------------------------------------------------

PS4 MATURE      (   500) neadatlvrqwqsggnnqvqaaagastsgsf
This G4 MATURE  (   500) -----------------------------
```

2

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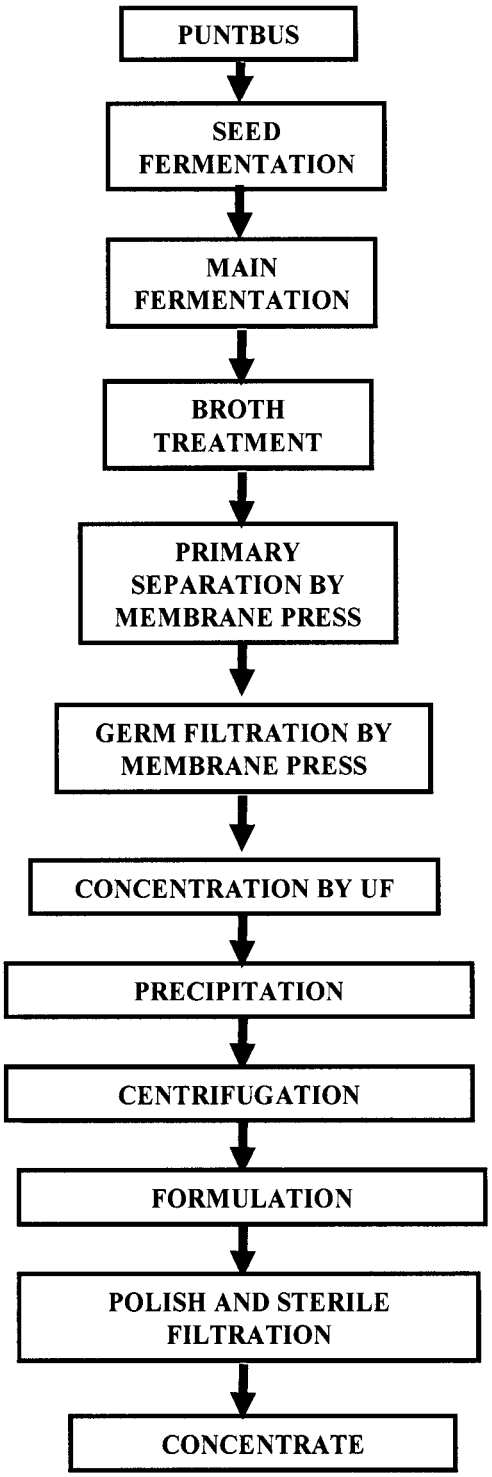
Appendix 2- The Manufacturing Process



3

Appendix 3 – Analysis of Safety Based on Pariza/Johnson Decision Tree

Pariza and Johnson have published guidelines for the safety assessment of microbial enzyme preparations (2001). These guidelines are based upon decades of experience in the production, use and safety evaluation of enzyme preparations. The safety assessment of a given enzyme preparation is based upon an evaluation of the toxigenic potential of the production organism. The responses below follow the pathway indicated in the decision tree. The outcome of this inquiry is that this G4-amylase enzyme preparation is "ACCEPTED" as safe for its intended use.

1. **Is the production strain genetically modified?** Yes, go to 2.

2. **Is the production strain modified using rDNA techniques?** Yes. The donor of this G4-amylase gene is *Pseudomonas saccharophila* and the host is *Bacillus licheniformis*. Go to 3a.

3a. **Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food?** Yes. Three previous generations of the enzyme, produced in *Bacillus subtilis*, have been determined to be GRAS. Furthermore, amylases with the designation 3.2.1.1, which are produced from fungal, bacterial and cereal sources, are widely used in the food industry and have been so for decades. Maltotetraohydrolases (EC 3.2.1.60) from *Pseudomonas stutzeri* strains, which are closely related to the three generations of this G4-amylase, have been used for producing maltotetraose and malto-tetraose syrups for use in foods since the 1980s[25, 32,-33]. Maltotetraose-forming amylase from *P. stutzeri* is listed as a natural additive in the Korean Food Additives Code. Go to 3c.

3c. **Is the test article free of transferable antibiotic resistance gene DNA?** Yes. Transformable DNA was not detected at or above the limit of 5 ng/ml in the enzyme preparation manufactured using this host and production process. Go to 3e.

3e. **Is all other introduced DNA well characterised and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?** Yes. The only DNA introduced is the endogenous *B. licheniformis* cat gene and this G4-amylase gene. The inserted sequences do not code for any known toxins. Go to 4.

4. **Is the introduced DNA randomly integrated into the chromosome?** No. The construct is designed to integrate into the *cat* locus by Campbell type recombination Go to 5.

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

was employed? Yes. Given the targeted integration (see 4 above) there is no concern for pleiotropic effects. Go to 6.

6. **Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?** Yes. The *B. licheniformis* host and methods of integration of the enzyme gene have been used by Genencor for production of many enzyme production organisms. Toxicology testing on 5 products from this strain lineage confirms the safety of the lineage. **Accept.**

4

GENENCOR

Appendix 4 – GRAS Panel Report

GENENCOR

Michael W. Pariza Consulting, LLC
7102 Valhalla Trail
Madison, WI 53719

Michael W. Pariza, Member

March 7, 2008

Alice J. Caddow
Vice President, Regulatory
And Environmental Affairs
Genencor, A Danisco Division
925 Page Mill Road,
Palo Alto, CA 94304

Dear Ms. Caddow:

I have reviewed the information you provided on Danisco's alpha-amylase SAS3 enzyme preparation expressed in a genetically modified strain of *Bacillus licheniformis* Bra 7. In this evaluation, I considered the biology and the safe lineage of the genetically-modified *Bacillus licheniformis* Bra 7 production organism, information provided by Danisco on the safety of the modified gene for alpha-amylase SAS3 and its expression product (the alpha-amylase SAS3 protein), and information regarding the gene source organism which has been reclassified as *Pseudomonas stutzeri* IAM1504 based on 16s rDNA sequence analysis (it had been previously misclassified as *P. saccharophilia* IAM1504 based on older biochemical/physiological assessment methodology).

Alpha-amylase enzyme preparations designated D34 and SAS2 that are substantially equivalent to SAS3 were previously determined to be GRAS by scientific procedures, as was SAS3 expressed in *B. subtilis*. The genes for D34 and SAS2 were both derived from *Pseudomonas stutzeri* IAM1504 and cloned into a strain of *Bacillus subtilis* that has a long history of safe use in food enzyme manufacture. SAS3 differs from the SAS2, D34, and *Pseudomonas stutzeri* IAM1504 wild-type amylase (designated PS4wt) catalytic core, by the number of amino acid modifications introduced into the amylase gene. These mutations were introduced to enhance stability and catalytic activity, thereby improving enzyme performance in baking applications.

The *B. licheniformis* Bra 7 production organism, which has been genetically modified to

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express the alpha-amylase SAS3 gene, has a long history of safe use in food enzyme manufacture. It was derived from a *B. licheniformis* safe strain lineage that has been previously described in GRAS assessment documents. These documents were reviewed by expert scientific panels, and determined to be GRAS by scientific procedures for food enzyme manufacture.

Based on these considerations, I conclude that the Danisco alpha-amylase SAS3 enzyme preparation derived from a genetically modified strain of *Bacillus licheniformis* Bra 7 by the manufacturing procedure you have described is GRAS for use in baking applications under the intended conditions of use that you have described.

Please note that this is a professional opinion directed at safety considerations only and not an endorsement, warranty, or recommendation regarding the possible use of the subject product by you or others.

Sincerely,

(b)(6)

Michael W. Pariza
Wisconsin Distinguished Professor of
Food Microbiology and Toxicology
Member, Michael W. Pariza Consulting LLC

RECEIVED
MAR 1 2 2009
BY: (b)(6)

GENENCOR

AM

Danisco US Inc
925 Page Mill Road
Palo Alto CA 94304
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Fax +1 650 845 6505
www.genencor.com

March 12, 2009

Dr. Robert Martin
Office of Food Additive Safety, HFS-255
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

RE: GRSN 277

Dear Dr. Martin:

Please accept the enclosed amendment to the above referenced GRAS Notice.

If you have questions or require additional information, please contact me at email: alice.caddow@danisco.com, phone: 650-846-7557 or fax at 650-845-6505.

Sincerely,

(b)(6)

Alice J. Caddow
Vice President of Regulatory
And Environmental Affairs

Enclosures (3 binders)

RECEIVED
MAR 13 2009
BY: (b)(6)

G4-amylase Enzyme Preparation from *Bacillus licheniformis* Expressing a Modified Maltotetraohydrolase (G4-amylase) Gene from *Pseudomonas stutzeri* Is Generally Recognized As Safe For Use in Food Processing

Notification Submitted by Genencor, a Danisco Division

January 7, 2009
(Amended March 10, 2009)

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1. GENERAL INTRODUCTION

The G4-amylase enzyme preparation under consideration is produced by submerged fermentation of *Bacillus licheniformis* carrying the gene encoding a protein engineered variant of the maltotetraohydrolase enzyme from *Pseudomonas stutzeri*. The *mta* gene encoding the native maltotetraohydrolase PS4wt from *P. stutzeri* was cloned by PCR based on the mta sequence[1]. To avoid heterogeneity of the G4-amylase upon limited proteolysis, the starch-binding domain was removed by introducing a stop codon after residue G429.

Subsequently, this G4-amylase has been developed by successive cycles of random, site specific mutagenesis and site scanning mutagenesis combined with screening for thermostability and exo-specificity. This G4-amylase contains sixteen amino acid changes compared to the sequence of the catalytic core of PS4wt.

By introducing these mutations, the temperature stability and baking performance of the enzyme is increased, making this G4-amylase far better suited for anti-staling applications than the wild-type maltotetraohydrolase enzyme from *P. stutzeri*.

This G4-amylase will be used to delay the staling process in bread and other baked goods and thereby extend the period for which the products have an acceptable eating quality. This is a benefit to the bread producers and to consumers, as bread will keep fresh for a longer time and disposal of stale bread and baked goods is minimized.

The systematic name of the principle enzyme activity is 4-α-D-Glucan maltotetraohydrolase. Other names used are glucan 1,4-α-maltotetraohydrolase, exo-maltotetraohydrolase, maltotetraohydrolase, maltotetraose-forming amylase, and G4-amylase.

The enzyme catalyses hydrolysis of 1,4-α-D-glucosidic linkages in amylaceous polysaccharides to remove successive maltotetraose residues from the non-reducing chain ends.

The EC number of the enzyme is 3.2.1.60, and the CAS number is 37288-44-1.

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. 170.36, Danisco has determined that its G4-amylase enzyme preparation from a modified strain of *B. licheniformis* expressing a protein engineered maltotetraohydrolase enzyme from *P. stutzeri* is a Generally Recognized As Safe ("GRAS") substance for the intended food application and is, therefore, exempt from the requirement for premarket approval. The information provided in the following sections is the basis of our determination of GRAS status of this G4-amylase enzyme preparation produced by a *B. licheniformis* host, which has been modified to express a gene encoding a protein engineered maltotetraohydrolase enzyme from *P. stutzeri*.

Our safety evaluation in Section 7 includes an evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure to the preparation.

The safety of the production organism must be the prime consideration in assessing the safety of an enzyme preparation intended for food use.[2,3] The safety of the production organism for this G4-amylase, *B. licheniformis,* is discussed in Sections 2 and 7. Another essential aspect of the safety evaluation of enzymes derived from genetically modified microorganisms is the identification and characterization of the inserted genetic material.[4-9] The genetic modifications used to construct this production organism are well defined and are described in Section 2. The safety evaluation described in Section 7 shows no evidence to indicate that any of the cloned DNA sequences and incorporated DNA code for or express a harmful toxic substance.

1.1 Name and Address of Notifier

Genencor, A Danisco Division
925 Page Mill Road
Palo Alto, CA 94304

1.2 Common or Usual Name of Substance

G4-amylase enzyme preparation from *B. licheniformis* expressing the gene encoding a protein engineered variant of the maltotetraohydrolase enzyme from *Pseudomonas stutzeri.*

1.3 Applicable Conditions of Use

This G4-amylase will be used to delay the staling process in bread and other baked goods and thereby extend the period for which the products have an acceptable eating quality. This is a benefit to the bread producers and to consumers as bread will keep fresh for a longer time and disposal of stale bread and baked good is minimized.

1.4 Basis for GRAS Determination

This GRAS determination is based upon scientific procedures.

1.5 Availability of Information for FDA Review

A notification package providing a summary of the information which supports this GRAS determination is enclosed with this notice. The package includes a safety evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure. Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying upon request.

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The production organism of the G4-amylase enzyme preparation is *B. licheniformis* strain GICC03279. It is derived by recombinant DNA methods from a strain of *B. licheniformis* modified to express a modified G4-amylase gene DNA (see Section 2.2). *Bacillus licheniformis* is considered a Class 1 Containment Agent under the NIH Guidelines for Research Involving Recombinant DNA Molecules and by the Ministerie van de Vlaamse Gemeenschap in Flanders, Belgium, and is also considered as GILSP worldwide. It also meets the criteria for a safe production microorganism as described by Pariza and Johnson[2] and several expert groups.[3-9] It

contains the modified G4-amylase gene under the regulation of a native *B. licheniformis* promoter and terminator along with a selectable marker, the native *B. licheniformis cat* gene.

The inserted DNA was integrated into the host on a in a vector derived from *Bacillus* plasmids pUB110 and pE194. The introduced DNA integrated into the Bacillus chromosome at the *cat* locus by Campbell type recombination. After integration all vector sequences of the plasmid were deleted by recombination between direct repeated *cat* sequences.

2.2 Host Microorganism

The host organism is *B. licheniformis* BRA7. *B. licheniformis* BRA7 was modified through deletion of several enzyme activities (proteases, amylase), a sporulation gene and the native chloramphenicol resistance genes to make it suitable for expression of G4-amylase. This strain lineage has been used by Genencor as a host for the commercial production of a number of α-amylases for the starch liquefaction business since 1989, as well as for production of protease, pullulanase, xylanase, and glycerophospholipid cholesterol acyltransferase. The strain has a sporulation frequency of less than 10^{-8} as determined by comparing the titer of the colony forming units (CFU) in the culture before and after heating at 85° C for 10 minutes. The strain, which has a history of safe large-scale fermentation, has been typed as *B. licheniformis* based on 16S rDNA gene sequencing and ribotyping.

2.3 G4-amylase Expression Vector

The vector used in this construction contains the following features: a temperature sensitive origin of replication (*ori* pE194, for replication in *Bacillus*), *ori* pBR322 (for amplification in *Escherichia coli*), the pUB110 neomycin resistance gene (*neo*) for initial selection, and the native *B. licheniformis* chloramphenicol resistance gene (*cat*) for selection, chromosomal integration and cassette amplification. The *cat* gene is present on a larger native *B. licheniformis* chromosomal fragment surrounding the coding sequence with upstream and downstream sequences. Part of the upstream *cat* sequence (called 5' repeat) is present twice on the plasmid to allow amplification of the expression cassette on the chromosome. The expression cassette (containing a native promoter and signal sequence and the G4-amylase gene) is located between these repeats.

The genetic construction was evaluated at every step to assess the incorporation of the desired functional genetic information and the final construct was verified by Southern blot analysis to confirm that only the intended genetic modifications to the *B. licheniformis* strain had been made.

2.4 Stability of the Introduced Genetic Sequences

The production strain proved to be 100% stable after at least 60 generations of fermentation, judged by chloramphenicol resistance and G4-amylase production.

2.5 Antibiotic Resistance Gene

The construction of the production organism utilized the native chloramphenicol acetyltransferase (*cat*) gene from *B. licheniformis* conferring chloramphenicol resistance; no new antibiotic resistance was introduced.

2.6 Absence of Production Microorganism in Product

The absence of the production microorganism is an established specification for the commercial product at a detection limit of 1CFU/g. The production organism does not end up in food and therefore, the first step in the safety assessment as described by the IFBC[4] is satisfactorily addressed.

3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1 Enzyme Identity

Classification	Glycoside hydrolase
IUBMB Nomenclature	4-α-D-Glucan maltotetraohydrolase
IUBMB Number:	3.2.1.60
CAS Number:	37288-44-1
Reaction catalyzed	hydrolysis of 1,4-α-D-glucosidic linkages in amylaceous polysaccharides to remove successive maltotetraose residues from the non-reducing chain ends.

3.2 Amino Acid Sequence

The G4-amylase in question is a variant of the wild type maltotetraohydrolase (PS4wt) from *P. stutzeri* strain IAM 1504 (obtained from the Institute of Applied Microbiology (IAM) Culture Collection, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Yayoi, Bunkyo-Ku, Tokyo 113-0032, Japan). The donor strain was obtained as *Pseudomonas saccharophila* strain IAM 1504. However, a recent Genencor International study reveals that *P. saccharophila* IAM 1504 should be reclassified as *P. stutzeri* IAM 1504, based on 16S rDNA sequencing and a screening program showing that the maltotetraohydrolase nucleotide sequence is restricted to the *P. stutzeri* cluster of strains. *P. stutzeri* is a non-fluorescent species belonging to rRNA similarity group I, while *P. saccharophila* belongs to group III (Palleroni[37] and references cited therein). The taxonomy of the Pseudomonads has recently been reviewed[38] based on 16S rRNA sequence.

Compared to the wild type enzyme PS4wt encoded by the gene *mta* of *P. stutzeri* strain IAM 1504, the starch-binding domain has been removed and 16 out of the remaining 429 amino acids of the catalytic core have been changed in order to improve thermostability and baking performance of the enzyme.

The amino acid sequence of this G4-amylase is shown in Appendix 1.

3.3 G4-amylase Donor Safety

Due to the recent reclassification of the donor species, the safety of both *Pseudomonas saccharophila* and *Pseudomonas stutzeri* are discussed herein. Both are gram negative, rod shaped bacteria mobile by means of a single polar flagellum. They are obligate aerobes, except in media with nitrate. They do not require organic growth factors and are found in soil and water[10].

Even though it is only the gene encoding the maltotetraose forming amylase which is transferred from the donor organism to the host organism, the safety of the donor organism was assessed, as *P. stutzeri* and *P. saccharophila* do not have a long history of safe use in the food industry. The safety was assessed based on search in the scientific literature.

The databases Medline, Chemtox and Chemical Abstract were searched. As the donor organism was identified as *P. saccharophila* by IAM, but as *P. stutzeri* based on 16s rDNA analysis, both the words "Pseudomonas" and "stutzeri or saccharophila" were used. These were combined with "pathogen or patogen or allergen or allergic or allergy or toxin or toxic or toxicology" for the period 1980 to 26 June 2006.

Reisler and Blumberg[11] reviewed the literature in the English language in the period January 1966 through August 1998 and reported 29 cases of *P. stutzeri* infections in humans. Among these cases were 2 deaths. Both patients were immunocompromised and in one case the death may not be directly related to the initial infection. They reported one case of community-acquired vertebral osteomyelitis caused by *P. stutzeri* and described it to be the first recorded case of a community-acquired, atraumatic infection in a previously healthy, non-immunocompromised person. The patient was successfully treated with antibiotics.

The database search revealed 3 other published papers on human infection cases with *P. stutzeri* (Campos-Herrero *et al*[12]; Potvliege *et al*[13] Puzenat *et al*[14]. All 3 patients were cured for their *P. stutzeri* infection by antibiotics. Two of the cases were included in the Reisler and Blumberg review, while one was reported more recently.

Lin *et al*[15] reported the isolation of *P. stutzeri* from the respiratory tract or bone marrow of dead chickens amongst 12 other Pseudomonas species and two other gram-negative hemolytic bacteria. Of the 15 species, 7 caused more than 50% mortality in chickens in an experimental set-up, while *P. stutzeri* resulted in less than 30% mortality. All strains including *P. stutzeri* were susceptible to some antibiotics and resistant to others.

Cortese *et al*[16] reported that *P. stutzeri* strain KC contains the MoeZ gene, which is essential for the synthesis of pyridine-2,6-bis(thiocarboxylic acid) (pbtc), a potential pathogenicity factor. However, other *P. stutzeri* strains and other Pseudomonas species tested lacked the pbtc synthesis genes. Cortese *et al*[17] showed that *P. stutzeri* strain KC and *P. putida* DSM301 are able

to produce pbtc, which is involved in the overall level of iron uptake, but they do not present any correlation to potential pathogenicity. Sebat *et al*[18] also describes *P. stutzeri* strain KC as a pbtc-forming organism and the strain demonstrated antagonism towards *E. coli*.

Martins *et al*[19] reports that *P. stutzeri* in some papers has been reported as capable of producing paralytic shellfish toxins (PSTs), but that results of autonomous (not related to presence of dinoflagellates) production is controversial. From their results in this paper the researchers do not conclude that autonomous production of PSTs by Pseudomonas can be excluded, but they show that utmost care must be taken in interpreting results and that previous findings have been false positives. Baker *et al*[29] reports that they can not rule out the possibility that *P. stutzeri* strain SF/PS produces saxitoxins; however, their data clearly demonstrate that the strain accumulates at least five different fluorescent compounds that could be easily mistaken for the saxitoxin derivative GTX_4.

4. MANUFACTURING PROCESS

This section describes the manufacturing process for this G4-amylase which follows standard industry practice.[20-22] For a diagram of the manufacturing process, see Appendix 2. The quality management system used in the manufacturing process complies with the requirements of ISO 9001. The enzyme preparation is also manufactured in accordance with FDA's current Good Manufacturing Practices ("cGMP") as set forth in 21 C.F.R. Part 110.

4.1 Raw Materials

The raw materials used in the fermentation and recovery process for this G4-amylase concentrate are standard ingredients used in the enzyme industry.[20-22] All the raw materials conform to the specifications of the Food Chemicals Codex, 6th edition, 2008 ("FCC"),[23] except for those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal requirements have been made in line with FCC requirements and acceptability of use for food enzyme production. Danisco uses a supplier quality program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and are verified upon receipt.

The antifoam and flocculants used in the fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 24, 1998. The maximum use level of these antifoam in the production process is $\leq$0.15% and of the flocculants <1.5%.

4.2 Fermentation Process

The G4-amylase is manufactured by submerged straight-batch or fed-batch pure culture fermentation of the genetically modified strain of *B. licheniformis* described in Section 2. All equipment is carefully designed, constructed, operated, cleaned and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and

chemical control measures are taken and microbiological analyses are conducted periodically to ensure absence of foreign microorganisms and confirm production strain identity.

4.2.1 Production organism

A new lyophilized stock culture vial of the *B. licheniformis* production organism described in Section 2 is used to initiate the production of each batch. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

4.2.2 Criteria for the rejection of fermentation batches

Growth characteristics during fermentation are observed microscopically. Samples are taken from each fermentation stage (inoculum, seed and main fermenter) before inoculation, at regular intervals during growth and before harvest or transfer. These samples are tested for microbiological contamination by plating on a nutrient medium.

A fermentation batch is declared as 'contaminated' if colony forming units (CFU) of bacteria or fungi other than the production strain are present at levels $>10^3$CFU/ml.

If a fermentation batch is determined to be contaminated, it will be rejected if deemed appropriate. If the contamination is minor and determined to be from common non-pathogenic environmental microbes, the fermentation may be processed.

4.3 Recovery Process

The recovery process is a multi-step operation which starts immediately after the fermentation process and consists of both concentration and formulation processes.

4.3.1 Concentration process

The enzyme is recovered from the culture broth by the following series of operations:

1. Primary separation –centrifugation or filtration
2. Polish filtration – for removal of residual production strain organisms and as a general precaution against microbial degradation
3. Concentration – ultrafiltration
4. Precipitation
5. Centrifugation
6. Formulation
7. Polish and sterile filtration
8. Concentration.

4.3.2 Formulation and standardization process

The ultrafiltered concentrate is stabilized with sodium benzoate. The preserved ultrafiltered concentrate is then dried and agglomerated using any one of the common drying methods, such as spray drying, fluid bed agglomeration or fluid bed spray drier.

4.3.3 Quality control of finished product

The final G4-amylase liquid concentrate from *B. licheniformis* is analyzed in accordance with the general specifications for enzyme preparations used in food processing as established by the Joint FAO/WHO Expert Committee on Food Additives ("JEFCA")[24] in 2006 and the FCC[23]. These specifications are set forth in Section 5.

The dried product is tested for enzyme activity, Total Viable Count and Dry Matter. The other assays conducted on the liquid concentrate are not repeated for the dried product. The dried product also is in compliance with the general specifications for enzyme preparations used in food processing as established by JEFCA[24] in 2006 and the FCC[23].

5. COMPOSITION AND SPECIFICATIONS

5.1 Quantitative Composition

The liquid concentrate is stabilized with 1% potassium sorbate and tested to demonstrate that it meets the specifications in section 5.2.

The liquid concentrate is then mixed with microcrystalline cellulose and spray dried to produce a product which is sold as tablets or in powder blends, as follows:

Activity for powder for tablets	>1600 BMK/g
Activity for powder for powder blends	1000 - 1500 BMK/g
Dry Matter	>91.0%

Dry Matter is measured by determining the loss on drying after 4 hours at 105°C and expressing as percentage change in weight. The tableted products also contain microcrystalline cellulose, sorbitol, wheat starch and sodium chloride while the powders contain microcrystalline cellulose, wheat starch and sodium chloride or microcrystalline cellulose, calcium carbonate, citric acid and BHT, depending upon the product.

5.2 Specifications

The G4-amylase preparation conforms to the general and additional requirements for enzyme preparations as described in the FCC[23]. In addition, the G4-amylase preparation also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by JEFCA[24].

The following specifications have been established for the G4-amylase and apply to both the liquid and dry products:

(See next page)

Property	Method Number	Reference Method	Specification
ENZYME ACTIVITIES			
Amylase, betamyl units	Danisco/Genencor Method	150,000 BMU/g (liquid) 1000 - 1500 BMK/g (powder for blends) >1600BMK/g (tablets)	Amylase, betamyl units
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	800V	ISO 4833 - "Microbiology -General guidance for the enumeration of micro-organisms - colony count technique at 30°C" and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Not more than 50,000 CFU/g
Total Coliforms	810V	ISO 4832 - "General guidance for the enumeration of coliforms - colony count technique" and the FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Not more than 30 CFU/g
E. coli	819V	ISO 7251 - Microbiology - "General Guidance for Enumeration of Presumptive *Escherichia coli* - Most Probable Number Technique" and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Negative/25g
Salmonella	832V	Nordic Committee on Food Analysis; Salmonella Bacteria; Detection in Foods. No 71; 4th Edition; 1991 and FDA	Negative/25g

Property	Method Number	Reference Method	Specification
		Bacteriological Analytical Manual; 8th Edition; AOAC International	
Production strain	892V	Genencor Method	Negative by test
Antibacterial Activity	899V	FAO Food and Nutrition Paper: 25th Session of the Joint FAO/WHO Expert Committee on Food Additives; Geneva 1981; p317-318; Appendix A	Negative by test
OTHER ASSAYS			
Lead	603W-PB	AAS/ICP-AES method in Jecfa, Combined Compendium of Food Additive Specifications, Volume 4, Rome, 2006	Less than 5 mg/kg

Lead and antibacterial activity are analyzed at regular intervals. Activity and microbial specifications are analyzed on every production batch of enzyme.

The lead, Coliforms and Salmonella specifications meet FCC and JEFCA requirements. The *E. coli* and antibacterial activity specifications meet JEFCA requirements and are not included in FCC. The production microorganism specification is a Danisco specification and is not mentioned in FCC or JEFCA.

The G4-amylase assay is colorimetric and monitors the rate of degradation of p-nitrophenyl maltoheptoside. Alpha-glucosidase and a glucoamylase are used as coupling enzymes. This substrate has the non-reducing terminal sugar chemically blocked, thus preventing any interaction with glucoamylase. The rate of p-nitrophenyl release is proportional to amylase activity and is monitored at 410nm. Note that this procedure involves a stop reaction at alkaline pH. The alkaline pH is necessary to stop the reaction and to provide optimal color development for the endpoint reaction.

The activity of Amylase SAS3 is defined in BMU units (liquid) and BMK units (dry). One BMK unit equals 1000 BMUs. This unit is not defined in exact terms, but relies on a specific assay and a standard enzyme. The assay is based on the enzyme's ability to break down blocked p-nitrophenyl maltoheptaoside. The specific activity of Amylase SAS3 is 3.0 BMU/mg enzyme protein using this assay.

6. APPLICATION

6.1 Mode of Action

There are two main applications of amylases in baking. The first is flour standardization, *i.e.* optimization of α-amylase activity in the flour, and the second is anti-staling.

Fungal α-amylase (EC 3.2.1.1) from *Aspergillus oryzae* is added to flour to optimise α-amylase activity. Use of fungal α-amylase in bread baking was approved in 1955 in the US and fungal α-amylase has since become the most widely used enzyme in baking world wide. Danisco started the marketing of fungal enzymes containing fungal α-amylase and xylanase activities to the milling and baking industry back in the 1980s.

The second important objective for use of amylases in baking is anti-staling, i.e., extending the freshness of baked goods. Staling is a highly complex phenomenon with firming being the most well-known and important symptom. Anti-staling amylases have to modify starch during the baking process to provide significant anti-staling effects. As intact starch granules are hardly attacked anti-staling amylases have to be sufficiently heat-stable to be active during the baking step after initial gelatinization of starch granules.

Endo-amylases (EC 3.2.1.1) are used for anti-staling and work by degrading amylose so that a weaker amylose network is formed during starch retrogradation after baking. Exo-amylases such as maltogenic amylase (EC 3.2.1.133) cloned from *Bacillus stearothermophilus* and now maltotetraohydrolase (EC 3.2.1.60) cloned from *P. stutzeri* offer clear improvements for anti--staling applications. By shortening the amylopectin side chains and releasing maltooligosaccharides, they efficiently reduce staling by lowering the rate of amylopectin retrogradation without disadvantageous side effects caused by excessive weakening of the amylose network.

To our knowledge, maltotetraohydrolase (EC 3.2.1.60) from *P. stutzeri* has only been used in baking via two Danisco products which were pre-cursors to this G4-amylase. Closely related maltotetraohydrolases (EC 3.2.1.60) from *P. stutzeri* strains have been used for producing maltotetraose and maltotetraose syrups marketed for use in foods since the 1980s[25]. Hayashibara Company Ltd markets a maltotetraose syrup called Tetrup which is produced using maltotetraohydrolase (http://www.hayashibara.co.jp/eng/contents_pdf.html) and holds patent EP289138B1 on maltotetraose-producing amylase from e.g. *P. stutzeri* and its use for maltotetraose production.

Maltotetraohydrolase (EC 3.2.1.60) belongs, like fungal α-amylase from *A. oryzae* (EC 3.2.1.1) and maltogenic amylase (EC 3.2.1.133), to the so-called α-amylase family or glycoside hydrolase family 13. It is a large enzyme family constituting many different reactions and product specificities (http://afmb.cnrs-mrs.fr/CAZY/) and sharing a common $(ß/α)_8$- or TIM-barrel catalytic domain. It consists of a closed eight-stranded parallel ß-sheet surrounded by eight α-helices. Four short conserved sequence motifs with seven residues strictly conserved in the α-amylase family are involved in forming the active site at the C-terminal ends of the ß-strands in

the $(\beta/\alpha)_8$-barrel. These four conserved sequence regions of the α-amylase family enzymes contain the three conserved catalytic residues Asp, Glu and Asp.

CAZy Family	Glycoside Hydrolase Family 13
Known Activities	α-amylase (EC 3.2.1.1); pullulanase (EC 3.2.1.41); cyclomaltodextrin glucanotransferase (EC 2.4.1.19); cyclomaltodextrinase (EC 3.2.1.54); trehalose-6-phosphate hydrolase (EC 3.2.1.93); oligo-α-glucosidase (EC 3.2.1.10); maltogenic amylase (EC 3.2.1.133); neopullulanase (EC 3.2.1.135); α-glucosidase (EC 3.2.1.20); maltotetraose-forming α-amylase (EC 3.2.1.60); isoamylase (EC 3.2.1.68); glucodextranase (EC 3.2.1.70); maltohexaose-forming α-amylase (EC 3.2.1.98); branching enzyme (EC 2.4.1.18); trehalose synthase (EC 5.4.99.16); 4-α-glucanotransferase (EC 2.4.1.25); maltopentaose-forming α-amylase (EC 3.2.1.-) ; amylosucrase (EC 2.4.1.4) ; sucrose phosphorylase (EC 2.4.1.7); malto-oligosyltrehalose trehalohydrolase (EC 2.4.1.141); isomaltulose synthase (EC 5.4.99.11).
Mechanism	Retaining
Catalytic Nucleophile/Base	Asp (experimental)
Catalytic Proton Donor	Glu (experimental)
3D Structure Status	Available (see PDB). Fold $(\beta/\alpha)_8$

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The enzyme preparation will be used in bakery products such as bread, bread buns, tortillas and crackers to delay the staling of these bakery products.

6.2 Use Levels

To obtain the desired anti-staling effects of this G4-amylase, the recommended dose is 2.0-30.0 mg enzyme protein/kg flour (2.28 – 34.2 mg TOS/kg flour).

6.3 Enzyme Residues in the Final Foods

The enzyme is largely inactivated during baking and has no further technical effect after baking.

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

The safety of the production organism must be the prime consideration in assessing the safety of an enzyme preparation intended for use in food. [3] If the organism is non-toxigenic and non-pathogenic, then it is assumed that foods or food ingredients produced from the organism, using current Good Manufacturing Practices, are safe to consume.[4] Pariza and Foster[3] define a non-toxigenic organism as 'one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure' and a non-pathogenic organism as 'one that is very unlikely to produce disease under ordinary circumstances.' *B. licheniformis* meets these criteria for non-toxigenicity and non-pathogenicity.

7.1.1 Safety of the host

Bacillus licheniformis is considered a Class 1 Containment Agent under the NIH Guidelines for Research Involving Recombinant DNA Molecules and by the Ministerie van de Vlaamse Gemeenschap in Flanders, Belgium, and is also considered as GILSP worldwide.

Mixed carbohydrase and protease from *B. licheniformis* were affirmed as GRAS by FDA on January 4, 1983 (48 FR 239). Also the FDA has no questions to four GRAS notices on enzymes derived from *B. licheniformis*:

- Alpha-amylase derived from *B. licheniformis* carrying a gene encoding a modified alpha-amylase derived from *B. licheniformis* and *Bacillus amyloliquefaciens* (GRN No. 22),
- Alpha-Amylase derived from *Bacillus licheniformis* carrying a gene encoding alpha-amylase from *B. stearothermophilus* (GRN No. 24),
- Pullulanase derived from *B. licheniformis* carrying a gene encoding pullulanase from *Bacillus deramificans*(GRN No. 72), and
- Alpha-amylase derived from *B. licheniformis* carrying a gene encoding a modified alpha-amylase from *B. licheniformis* (GRN No. 79).

Amylase from *B. licheniformis* has been reviewed by the Joint Expert Committee on Food Additives (JECFA) of FAO/WHO and an acceptable daily intake (ADI) "not specified" has been set[26].

B. licheniformis , including genetically modified strains, has been approved for the production of amylase enzymes in the food industry in France and it is also approved for the production of proteases and pullulanase (Arrêté du 05/09/1989 et compléments).

Strains of *B. licheniformis* are found in Table V of Division 16 of "Canadian Food and Drugs Act and Regulations", as an authorized source for amylases and proteases in several food applications.

The species *B. licheniformis* is an accepted source of safe food enzymes in the literature. The safety of *B. licheniformis* strains was recently reviewed by Priest *et al*[27]. It is also recognized as a safe host strain for recombinantly derived enzymes by Olempska-Beer *et al*[28].

Pathogenic strains of *B. licheniformis* are NOT described in the Bergey Manual[10] or in the ATCC and other catalogues. The species *B. licheniformis* does not appear on the Proposal for a Council Directive amending the "Directive 90/679/EEC on the protection of workers from risks related to exposure to biological agents at work".

Strains of *B. licheniformis* are found in the Sixth edition of "Food Chemicals Codex" as a source for the enzymatic preparation of carbohydrase and protease used in the treatment of foods.

The parent strain of the current production strain and its progeny, *B. licheniformis* BRA7, have been used by Genencor for the production of α-amylase enzyme preparations since 1989, as well as for the production of proteases, pullulanase and xylanase.

Numerous oral toxicity mutagenicity and carcinogenicity studies using enzyme products from *B. licheniformis* BRA7-derived strains have been performed, and no evidence of a toxic or mutagenic effect has been observed.

7.1.2 Safety of the donor source

See section 3.3.

7.2 Safety of the Manufacturing Process

The manufacturing process for the production of G4-amylase will be conducted in a manner similar to other food and feed production processes. It consists of a pure-culture fermentation process, cell separation, concentration, filtration and formulation, resulting in a liquid G4-amylase enzyme concentrate that is then dried and agglomerated for use in baking. The process, described in Section 4, is conducted in accordance with FDA's cGMPs as set forth in 21 C.F.R. Part 110. The resultant products meet the general requirements for enzyme preparations of the FCC[23] and WHO/JEFCA[24].

7.3 Safety of G4-amylase

7.3.1 Allergenicity

According to Pariza and Foster[3], there have been no confirmed reports of allergies in consumers caused by enzymes used in food processing. Amylases have been used extensively in food processes for over 50 years and have generated no known safety concerns. It is well-known that occupational exposure to airborne flour dust and enzyme particles can lead to dermal and respiratory sensitization among industry workers (AMFEP, 1998). The rate of such occurrences is relatively low, however. Studies have shown that occupational exposure does not give rise to allergic responses among workers or consumers upon ingestion of enzyme-treated baked goods. For example, Cullinan *et al*[30] studied 17 adults with demonstrated sensitivity to *Aspergillus* species (positive skin prick response). Subjects were fed bread containing *Aspergillus*-derived α-amylase or hemicellulase and an enzyme-free control bread on consecutive days. There were no consistent clinical or functional reactions to active challenge in any of the subjects.

In 1998 the Association of Manufacturers of Fermentation Enzyme Products (AMFEP) Working Group on Consumer Allergy Risk from Enzyme Residues in Food reported on an in-depth analysis of the allergenicity of enzyme products. They concluded that there are no scientific indications that small amounts of enzymes in bread and other foods can sensitize or induce allergy reactions in consumers, and that enzyme residues in bread and other foods do not represent any unacceptable risk to consumers. Further, in a recent investigation of possible oral allergencity of 19 commercial enzymes used in the food industry, there were no findings of clinical relevance even in individuals with inhalation allergies to the same enzymes, and the authors concluded "that ingestion of food enzymes in general is not considered to be a concern with regard to food allergy[31]."

Despite this lack of general concern, the potential that G-4 amylase could be a food allergen was examined. FASTA alignments of the full SAS3 sequence with known allergens using either the SDAP database (http://fermi.utmb.edu/SDAP/sdap_who.html)containing 737 allergens or the Allermatch

database combined database (www.allermatch.org) containing 792 allergens suggests a low degree of homology (approx. 20%, E score 10^{-8}) with Asp o 21 (*Aspergillus oryzae* TAKA amylase a, sequences AAA32708 and BAA00336 in Genbank and described in Tsukagoshi et al.[34]). The low degree of homology in itself does not imply SAS3 to be a food allergen.

Following the recommendations in FAO/WHO[35], two additional types of searches against the combined Allermatch database containing non-redundant allergen sequences from the SwissProt database and the WHO-IUIS list were performed using Allermatch software (Fiers et al., [36]) as well as against the SDAP database were performed.

The initial search according to the FAO/WHO guidelines, using a sliding window of 80-amino acid stretches and greater than 35% identity to indicate a match, revealed no matches of SAS3 with known allergens. A secondary, more detailed search for exact matches of short stretches (6 amino acids) of sequence that could serve as potential IgE binding sites indicated that no such short matching stretches exist, including to the *A. orzyae* amylase, confirming that SAS3 is not a food allergen.

Using the FAO/WHO recommendations, we conclude that SAS3 is unlikely to be a food allergen.

7.3.2 Safety Studies

7.3.2.1 Test item

The UF Concentrate was prepared as the test item for the safety studies. UF concentrate is the ultrafiltered concentrate of the enzyme preparation, stopped during the manufacturing process before the addition of formulation ingredients. The UF concentrate is characterized by:

Enzyme activity	241318 BMU/g or 3000 BMU/mg enzyme protein
pH	4.93
Specific gravity	1.028
%TOS (100 –%ash-%moisture)	9.09%
Total Protein content	78.76 mg/ml

The UF concentrate was stored frozen in aliquots and thawed before use.

The test item was used to conduct the following studies:

Study
Acute dermal irritation study in rabbits (sequential approach)
Acute Eye Irritation/Corrosion Study in the Rabbit
Acute oral toxicity in rats – Fixed dose procedure
Sub-chronic 13 week toxicity in the rat

Bacterial Reverse Mutation Assay – Ames assay
In vitro chromosomal aberration Study.

7.3.2.2 Acute dermal irritation study in rabbits (sequential approach)

A. Procedure:

The objective of this study was to assess the local irritant effect of this G4-amylase. This study was conducted according to the method recommended in the OECD Guideline No. 404 "Acute dermal irritation/corrosion" (adopted 24 April 2002) and was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999) and the requirements of Directives 2004/9/EC and 2004/10/EC.

In the initial test, the back of one rabbit was shaved and three test sites were selected. At each test site a quantity of 0.5 ml of the test material was placed under a cotton gauze patch, which was secured in position with a strip of adhesive tape. One patch was removed at each of three time points: 3 minutes, 1 hour and 4 hours after application.

In the confirmatory test, two additional rabbits were treated with 0.5 ml of the test material. One patch was applied and was allowed to remain in contact for 4 hours. The test sites were examined for irritation approximately one hour following the removal of the patches and at 24, 48 and 72 hours later.

B. Results

No deaths or overt signs of toxicity were observed in this study. No effects on feed consumption and weight gain were recorded. No erythema, eschar or edema was observed at these test sites at any of the examinations throughout the study.

The test material produced a primary irritation index of 0.0 and no corrosive effects were noted.

C. Evaluation

On the basis of the results obtained in this study, this G4-amylase is classified as non-irritant according to OECD Guideline No 404, "Acute Dermal Irritation/Corrosion", April 2002, and the Official Journal of the European Communities, L 110 A, volume 36, 4 May 1993 § 3.2.5 ("C", R35 or R34) and § 3.2.6.1 ("Xi", R38).

7.3.2.3 Acute Eye Irritation/Corrosion Study in the Rabbit

A. Procedure

The objective of this study was to assess the ocular irritation potential of this G4-amylase. This study was conducted according to the method recommended in the OECD Guideline No. 405 "Acute eye irritation/corrosion" (adopted 24 April 2002) and was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999) and the requirements of Directives 2004/9/EC and 2004/10/EC.

In the initial test, the test material was applied at 0.1 ml to the conjunctival sac of the right eye. The left eye served as control. Assessment of ocular damage/irritation was made at approximately 1 hour and at 24, 48 and 72 hours after application. Examination of the eye was facilitated by the use of a light source from a standard ophthalmoscope. In the confirmatory test, two additional rabbits were used.

B. Results

One rabbit was killed for humane reasons, immediately after the 48-hour observation period, due to a broken front limb. As full recovery from ocular effects had already been noted by the time of its sacrifice, the absence of this rabbit was considered not to affect the purpose or integrity of the study.

No corneal or iridial effects were noted. Minimal to moderate conjunctival irritation was noted in all treated eyes one hour after treatment. At the 24-hour observation period, only minimal conjunctival irritation was noted. All eyes were cleared by the 48 and 72-hour periods. The test material produced a maximum group mean score (PIS = primary irritation score) of 7.3/110.0 at the 1 hour observation period, a PIS of 0.7/110.0 at the 48-hour observation period and a PIS of 0.0/110.0 at the 72-hour observation period.

C. Evaluation

The maximal primary eye irritation score was 7.3/110.0. According to the EEC Directive published in: EEC Directive published in: "Official Journal of the European Communities" No: L 383 A, volume 35, 29.12.1992, part B5: Acute toxicity (eye irritation) and No: L 110 A, volume 36, 04.05.1993, part 3.2.6.2 Ocular lesions (which is implemented in Commission Directive 2001/59/EC of 6 August 2001), this G4-amylase is classified as mild irritant.

7.3.2.4 Acute oral toxicity in rats – Fixed dose procedure

A. Procedure:

The objective of this study was to assess the acute toxicity of this G4-amylase when administered as a single oral dose followed by a 14-day period of observation. The information is used for both hazard assessment and ranking purposes. The study was initiated with a sighting study using 2000 mg/kg with one female Sprague Dawley CD rat. In the absence of toxicity at a dose level of 2000 mg/kg, an additional group of 4 female rats was dosed with 2000 mg/kg bw.

This study was conducted according to OECD Guideline No. 420 "Acute oral toxicity – Fixed dose method" (adopted 17 December 2001) and was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999) and the requirements of Directives 2004/9/EC and 2004/10/EC.

B. Results:

No mortality was recorded in this study. There were no overt signs of systemic toxicity throughout the 14-day observation period and at necropsy.

C. Evaluation:

Under the conditions of this study, the oral LD_{50} was equal or greater than 2000 mg/kg bw corresponding to GHS category 4 according to Annex 3 of the OECD Guideline for Testing of Chemicals No. 420. This G4-amylase is classified as "Practically non toxic by ingestion".

7.3.2.5 A 13-week Oral (Gavage) Toxicity Study in Rats

A. Procedure

The objective of this study was to investigate the potential of this G4-amylase to induce systemic toxicity after repeated daily oral administration to Wistar rats of both sexes for 90 consecutive days. Groups of 10 rats/sex each were gavaged daily with 0 (0.9% saline), 0, 23.7, 47.4 or 79 mg total protein/kg body weight in a constant volume of 5 ml 0.9% saline/kg body weight corresponding to 0, 27.3, 54.5 or 90.9 mg TOS/kg bw/day, respectively.

Animals of the same sex were housed three or four in solid floor polypropylene cages with softwood flake bedding (Harlan UK, Oxon) and had access to water (via bottle) and feed *ad libitum*. A pelleted diet (Rodent 2014C, Harlan UK, Oxon) was used. For environmental enrichment, the animals were provided a

supply of Aspen Wood Wool at each change of bedding. All groups were housed under controlled temperature, humidity and lightning conditions.

All animals were observed daily for mortality and signs of morbidity. Body weight and feed consumption were recorded weekly. Water consumption was recorded twice weekly for each cage. Ophthalmologic examination was performed on all animals prior to study initiation and in the control and high dose groups at study termination. Hematology was conducted on Day 0, 30 and 90. A functional observation battery consisting of detailed clinical observation, reactivity to handling and stimuli and motor activity examination was conducted during week 13 for all animals. Clinical chemistry was evaluated at study termination prior to necropsy on all groups. After a thorough macroscopic examination, selected organs were removed, weighed and processed for future histopathologic examination. Microscopic examination was conducted on selected organs from control and high dose animals. If a questionable finding was noted, the microscopic examination would be extended to the low and mid dose groups.

This study was conducted in accordance with OECD guideline No. 408 "Subchronic oral toxicity – Rodent: 90 day study" (adopted 21 September 1998), EPA Guideline OPPTS 870.3100 (August 1998) and was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999) and the requirements of Directives 2004/9/EC and 2004/10/EC.

B. Results

No treatment-related deaths were recorded throughout the entire investigation. One low dose female was killed *in extremis* on day 68 following signs of lethargy, pilo-erection, hunched posture and staining around the snout. These observations were considered as non-treatment related. One mid dose female was found dead on day 90 just prior to terminal kill. This animal did not show any clinical signs prior to death.

Weekly open-field arena observations did not reveal any treatment-related effects. No treatment-related changes in functional performance tests were detected. No treatment-related changes in sensory reactivities were detected in the treated groups as compared with controls. There were no biological or statistical differences between the control and treated groups with respect to body weights, feed consumption, water consumption, feed efficiency (ratio of body weight gain to dietary intake), hematology, and ophthalmologic examinations.

Females from all treated groups showed an increase in serum potassium in comparison to concurrent controls. On review of historical control data, these increases were attributed to lower than expected concurrent control values. The

increases in serum potassium were not statistically different from historical control values.

Females from all treated groups showed a decrease in absolute and relative ovary weights when compared to concurrent controls. On review of historical control data, the control values for this parameter were higher than the expected ranges for female rats of the age and strain employed. In the absence of histopathologic findings, these findings were not attributed to treatment. Incidental macroscopic findings were noted (one high dose male displayed small kidneys; one high dose male with hydronephrosis on the right kidney) but were not considered as treatment-related in the absence of histopathologic changes. Scattered histopathologic findings were noted, but all morphological changes were those commonly observed in rats of the age and strain employed and there were no differences in severity or incidence between control and treated groups.

C. Evaluation

There were 2 interim deaths in this study but both were considered as non-treatment related. Blood chemical changes were confined to females and consisted of an increase in serum potassium. Since the changes were within the range of the historical control data, they were not considered as treatment-related. Significant decreases in absolute and relative ovary weights were noted when compared to concurrent control values. However, on review of historical control data, the control values for this parameter were higher than the expected ranges for female rats of the age and strain employed. Therefore, these findings were considered as non-treatment related especially in the absence of histopathological changes in the ovaries.

It can be concluded that daily administration of G4-amylase by gavage for 90 consecutive days did not result in adverse systemic toxicity or adverse effects on clinical chemistry, hematology, functional observation tests and macroscopic and histopathologic examinations. Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established at the highest dose tested, 79 mg total protein/kg bw/day corresponding to 90.0 mg TOS/kg bw/day and 241318 BMU/kg bw/day.

7.3.2.6 Bacterial Reverse Mutation Assay – Ames assay

A. Procedure:

The objective of this assay was to assess the potential of G4-amylase to induce point mutations (frame-shift and base-pair) in four strains of *Salmonella typhimurium* TA 98, TA 100, TA 1535 and TA 1537 and *E. coli* strain WP2uvrA$^-$. The test material was tested both in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). The tests were

performed using the plate incorporation method, at 5 dose levels, in triplicate. The dose levels selected for the assay were based on the total protein content of the test material (1 ml contained 79 mg total protein).

A preliminary toxicity test was carried out with dose levels ranging from 0.15 to 5000 µg/plate.

The test material (0.1 ml) was mixed with 0.1 ml of bacterial suspension, 2 ml of molten agar, 0.5 ml of S-9 mix or phosphate buffer and overlaying onto sterile plates of agar. Sterile distilled water served as vehicle control. After 48 hours incubation at 37°C, the plates were assessed for the number of revertant colonies and growth of the bacterial background lawn.

In the main assay, five concentrations of the test material were assayed in triplicate against each tester strain in both the presence and absence of metabolic activation. The test material (0.1 ml) was mixed with 0.1 ml of bacterial suspension, 2 ml of molten agar, 0.5 ml of S-9 mix or phosphate buffer and overlaying onto sterile plates of agar. Sterile distilled water served as vehicle control. The positive controls used for plates without S-9 mix were N-ethyl-N'-nitro-nitrosoguanidine, 9-aminoacridine and 4-nitroquinoline-1-oxide. The positive controls used for plates with S-9 mix were 2-aminoanthracene and benzo(a)pyrene. After 48 hours incubation at 37°C, the plates were assessed for the number of revertant colonies and growth of the bacterial background lawn.

All dose levels were expressed in terms of total protein. The highest dose level tested (5000 µg/plate) is the maximum required by the OECD guideline. This assay was conducted in accordance with OECD guideline No. 471 "Bacterial reverse mutation test", the US EPA (TSCA) OPPTS harmonized guidelines, mutagenicity guidelines published by the Japanese Regulatory Authorities (METI, MHLW and MAFF) and was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999) and the requirements of Directives 2004/9/EC and 2004/10/EC.

B. Results:

In the screening assay, G4-amylase was not toxic to the test bacteria up to and including the highest dose level tested (5000 µg/plate) in both the presence and absence of S-9 mix. The test material caused no visible reduction in the growth of the bacterial background lawn at any dose level. Therefore, 5000 µg/plate was selected as the highest dose level for the main tests.

In the main tests, five dose levels (50, 150, 500, 1500 and 5000 µg/plate) were used. Insoluble material was observed at and above the 1500 µg/plate but this did not prevent the scoring of the revertant colonies. No biologically or statistically significant increases in the number of revertant colonies were observed in any

dose level in either main test. Statistical increases in the number of revertant colonies were noted with the positive controls in both the presence and absence of metabolic activation substantiating the sensitivity of the treat and plate assay and the efficacy of the metabolic activation mixture.

C. Evaluation

Under the conditions of this assay, G4-amylase has not shown any evidence of mutagenic activity in the Ames assay.

7.3.2.7 *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes

A. Procedure

The objective of this assay was to investigate the potential of G4-amylase to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). G4-amylase was mixed with cultures of human peripheral lymphocytes both in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver; S-9 mix). This assay consisted of a preliminary toxicity (dose range finding) assay and two main tests. Five concentrations of G4-amylase were used in the preliminary assay and at least 3 dose levels were then selected for the two main assays with the highest dose level clearly inducing a toxic effect (50% reduction in mitotic index). In the absence of cytotoxicity, the highest dose selected would be 5000 µg/ml, as recommended by the OECD guideline.

With metabolic activation:
Cells were grown in Eagle's essential medium with HEPES buffer (MEM) at 37°C with 5%CO2 in air. After 48 hours, the cell cultures were transferred to tubes and centrifuged. Approximately 9 ml of the culture medium was removed and G4-amylase (1 ml), S-9 mix (1ml), and MEM culture media (7ml) were added to the cell cultures and incubated at 37°C for 4 hours. The cultures were then centrifuged, the treatment medium removed and washed with MEM culture medium. After a further centrifugation, the MEM culture medium was removed by suction and replaced with the fresh MEM culture medium. The cell cultures were then re-incubated for an additional 20 hours at 37°C in 5% CO_2 in humidified air.

Without metabolic activation:
Cells were grown in Eagle's essential medium with HEPES buffer (MEM) at 37°C with 5%CO2 in air. After 48 hours, the cell cultures were transferred to tubes and centrifuged. Approximately 9 ml of the culture medium was removed and G4-amylase (1 ml) and MEM culture media

(8ml) were added to the cell cultures and incubated at 37°C for 4 hours. The cultures were then centrifuged, the treatment medium removed and washed with MEM culture medium. After a further centrifugation, the MEM culture medium was removed by suction and replaced with the fresh MEM culture medium. The cell cultures were then re-incubated for an additional 20 hours at 37°C in 5% CO_2 in humidified air.

All cultures (with and without S-9 mix) were harvested 24 hours after the start of treatment. Two hours prior to harvest, Demecolcine (0.1 μg/ml) was added to all cultures to arrest all cells at the metaphase-stage of mitosis. At the harvest time, all cultures were centrifuged and the supernatant discarded. The cell pellets were resuspended in a KCl solution, incubated for 10 minutes, centrifuged and the supernatant removed. The cells were then fixed on slides, stained and scored for chromosomal aberrations:

a. Cytotoxicity was evaluated using the mitotic index (number of cells in mitosis/1000 cells examined. From these results, a dose level causing a decrease in mitotic index of 50% was selected as the highest dose in the main tests.

b. Metaphase analysis (i.e. evaluation of chromosomal aberration) was conducted on 100 metaphases per dose level.

c. Mitomycin C and cyclophosphamide were used as positive controls for cultures without S-9 mix and with S-9 mix, respectively.

This assay was conducted in accordance with OECD guideline No. 473 "*In vitro* Mammalian chromosome aberration test" and was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999) and the requirements of Directives 2004/9/EC and 2004/10/EC.

B. Results

In the preliminary assay, the dose range was 19.53 to 5000 μg/ml. Cytotoxicity (only 6% survival) was noted at the 312.5 μg/ml dose level. Therefore, the maximum dose level selected for the 4-hour incubation period in the presence and absence of S-9 mix was 625 μg/ml and the maximum dose selected for the 24-hour continuous exposure was 312.5 μg/ml.

In both main assays, G4-amylase did not induce a statistically significant increase in the frequency of cells with aberrations either in the absence or presence of metabolic activation.

In both main assays, G4-amylase did not induce a statistically significant increase in the numbers of polyploid cells at any dose level either in the presence or absence of metabolic activation.

In both main assays, significant increases in aberrant metaphases were noted with the positive controls demonstrating the sensitivity of the tests and the efficacy of the S-9 mix.

C. Evaluation

Under the conditions of this test, G4-amylase did not induce chromosomal aberrations (both structural and numerical) in this *in* vitro cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation.

7. 4. Overall Safety Assessment and Human Exposure

According to the Directive of the Commission 93/21/EEC of April 27, 1993, G4-amylase is non hazardous based on acute oral and irritation studies. In genotoxicity studies, G4-amylase is not mutagenic, clastogenic or aneugenic. Daily oral administration of G4-amylase up to and including a dose level of 79 mg total protein/kg bw/day (90.0 mg TOS/kg bw/day) does not result in any manifestation of systemic, hematologic, functional or histopathologic adverse effects.

7.4.1 Identification of the NOAEL

In the 90-day oral (gavage) study in rats (SafePharm Lab No. 2420-0008), a NOAEL was established at 79 mg total protein/kg bw/day (equivalent to 90.0 mg TOS/kg bw/day). The study was conducted in compliance with both the UK and OECD Good Laboratory Practice Regulations and was designed based on OECD guideline No. 408. Since human exposure to G4-amylase is through oral ingestion, selection of this NOAEL is thus appropriate.

No Observed Adverse Effect Level = 79 mg total protein/kg bw/day
= 90.0 mg TOS/kg bw/day

7.4.2 Human Exposure Assessment

G4-amylase is used in bakery. Based on the Food Consumption Statistics collected from 1979-1988 by OECD (1991), Italy has the highest daily consumption of bread, i.e. 4.92 g/kg bw/day. Data from the USDA, Agricultural Research Service (1997) reveals the

maximum daily bread consumption in the US as 4.07 g/kg bw/day. Therefore, for the purpose of this safety assessment:

1. The maximum daily bread consumption of 4.92 g/kg bw/day is used to represent a worst-case scenario:
   Maximum daily bread consumption = 4.92 g/kg bw/day;
2. Since the flour content in bread is 66%, the maximum daily flour consumption is:
   4.92 g/kg bw/d X 66% = 3.25 g/kg bw/day;
3. G4-amylase is applied at the recommended range of 2.0 to 30.0 mg enzyme protein/kg flour;
4. Maximum intake of G4-amylase from flour:
   3.25 g/kg bw/day X 30 mg/kg flour = 0.098 mg enzyme protein/kg bw/day;
5. Daily exposure to G4-amylase (worst case) assuming that 100% of enzyme remains in the bread, not removed or inactivated by heat and also on the assumption of 100% market penetration =
   0.098 mg enzyme protein/kg bw/day (0.11 mg TOS/kg bw/day)

Risk Assessment

Risk for humans through ingestion of bakery made from flour treated with G4-amylase is measured by a Margin of Exposure (MOE), which determines how close the estimated daily human exposure comes to a No Observable Adverse Effect Level (NOAEL). Generally, a MOE greater than 100 means consumption is not of concern.

$$\text{Margin of exposure} = \frac{\text{No observed adverse effect level}}{\text{Daily exposure}}$$

Based on the results from the 90-day oral (gavage) feeding study cited above,

$$\text{Margin of exposure} = \frac{\text{79 mg enzyme protein/kg bw/day}}{\text{0.098 mg/kg bw/day}} = 806.$$

7.5 Conclusion

The safety of G4-amylase is assessed in a battery of toxicology studies investigating its acute oral, irritation, mutagenic and systemic toxicity potential. G4-amylase is not a skin irritant, is not acutely toxic by ingestion and is a mild eye irritant. A battery of genotoxicity assays was conducted and under the conditions of these assays G4-amylase is not a mutagen, a clastogen, or an aneugen.

Daily administration of G4-amylase by gavage for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at 79 mg total protein/kg bw/day (equivalent to 90.0 mg TOS/kg bw/day).

Even under the worst case scenario that G4-amylase is applied at the maximum rate (30 mg enzyme protein/kg flour) and the enzyme is not destroyed and/or removed during baking, the use

of G4-amylase as a food additive in bakery is not expected to result in adverse effects to humans. Based on the NOAEL of 79.0 mg total protein/kg bw/day (90.0 mg TOS/kg bw/day), a safety margin of 806-fold exists for the proposed uses.

8. BASIS FOR GENERAL RECOGNITION OF SAFETY

As noted in the Safety sections above, enzyme preparations derived from *B. licheniformis*, including α-amylase, protease, pullulanase, and xylanase, are well recognized by qualified experts as being safe. Published literature, government laws and regulations, reviews by expert panels such as FAO/WHO JECFA, as well as Genencor's own unpublished safety studies, support such a conclusion.

Bacillus licheniformis is widely used by enzyme manufacturers around the world for the production of enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. It is a known safe host for enzyme production. An enzyme preparation derived from a recombinant microorganism will be safe if the host microorganism is nonpathogenic and non-toxigenic; the genetic information that is introduced into the host microorganism is well characterized; and the added DNA does not encode and express any known harmful or toxic substances. Information is provided in this document to support that the components of the enzyme preparation meet these standards.

The safety studies conducted by Genencor established a NOAEL of 79.0 mg total protein/kg bw/day (90.0 mg TOS/kg bw/day) and a safety margin of 806-fold for the proposed uses.

Utilizing the information provided, the enzyme preparation was taken through the Pariza-Johnson decision tree for microbial enzymes (see Appendix 3) and the preparation was determined to be acceptable for use in foods as described above.

Based on the available data from the literature and generated by Genencor, the company has concluded that the enzyme preparation from *B. licheniformis* strain GICC03279 is safe and suitable for use in bakery products.

In addition, the safety determination including construction of the production organism, the production process and materials, and safety of the product were reviewed by Dr. Michael W. Pariza, who concurred with the company's conclusion that the product is GRAS (see Appendix 4) (note, the safety studies on this G4-amylase were conducted after the GRAS determination by Dr. Pariza to support regulatory approvals outside the US where toxicology testing is required).

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10. LIST OF APPENDICES

Appendix 1 – The amino acid sequence of the G4-amylase

Appendix 2 - The Manufacturing process

Appendix 3 – The Pariza/Johnson Decision Tree

Appendix 4 – GRAS Panel Report

1

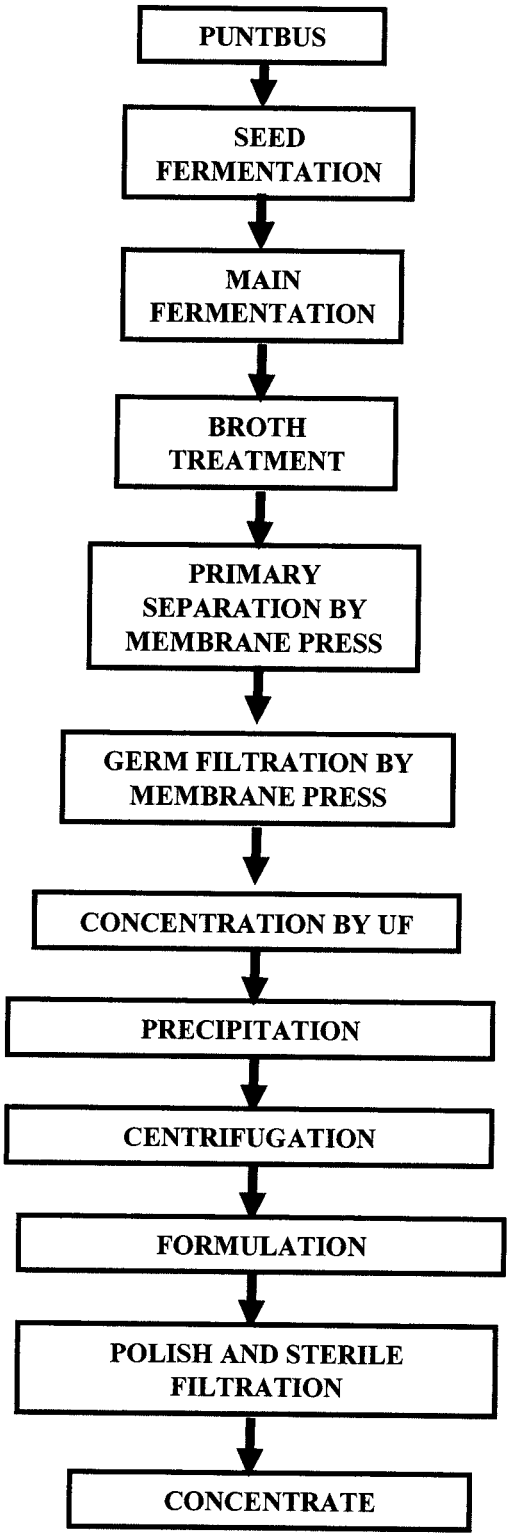
Appendix 1 – The amino acid sequence of this G4-amylase

Amino acid sequence alignment of this G4-amylase vs. PS4 wild type Maltotetraohydrolase. The amino acids residue positions changed between wild type PS4 and this G4-amylase are shown in green.

```
PS4 MATURE      (     1) -dqagkspagvryhggdeiilqgfhwnvvreapndwynilrqqastiaad
This G4 MATURE  (     1) ...................................................

PS4 MATURE      (    50) gfsaiwmpvpwrdfsswtdggksgggegyfwhdfnkngrygsdaqlrqaa
This G4 MATURE  (    51) ...................................................

PS4 MATURE      (   100) galggagvkvlydvvpnhmnrgypdkeinlpagqgfwrndcadpgnypnd
This G4 MATURE  (   101) ...................................................

PS4 MATURE      (   150) cddgdrfiggesdlntghpqiygmfrdelanlrsgygaggfrfdfvrgya
This G4 MATURE  (   151) ...................................................

PS4 MATURE      (   200) pervdswmsdsadssfcvgelwkgpseypswdwrntaswqqiikdwsdra
This G4 MATURE  (   201) ...................................................

PS4 MATURE      (   250) kcpvfdfalkermqngsvadwkhglngnpdprwrevavtfvdnhdtgysp
This G4 MATURE  (   251) ...................................................

PS4 MATURE      (   300) gqnggqhhwalqdglirqayayiltspgtpvvywshmydwgygdfirqli
This G4 MATURE  (   301) ...................................................

PS4 MATURE      (   350) qvrrtagvradsaisfhsgysglvatvsgsqqtlvvalnsdlanpgqvas
This G4 MATURE  (   351) ...................................................

PS4 MATURE      (   400) gsfseavnasngqvrvwrsgsgdgggndggegglvnvnfrcdngvtqmgd
This G4 MATURE  (   401) ...................................................

PS4 MATURE      (   450) svyavgnvsqlgnwspasavrltdtssyptwkgsialpdgqnvewkclir
This G4 MATURE  (   450) ---------------------------------------------------

PS4 MATURE      (   500) neadatlvrqwqsggnnqvqaaagastsgsf
This G4 MATURE  (   500) -----------------------------
```

2

Appendix 2- The Manufacturing Process



3

Appendix 3 – Analysis of Safety Based on Pariza/Johnson Decision Tree

Pariza and Johnson have published guidelines for the safety assessment of microbial enzyme preparations (2001). These guidelines are based upon decades of experience in the production, use and safety evaluation of enzyme preparations. The safety assessment of a given enzyme preparation is based upon an evaluation of the toxigenic potential of the production organism. The responses below follow the pathway indicated in the decision tree. The outcome of this inquiry is that this G4-amylase enzyme preparation is "ACCEPTED" as safe for its intended use.

1. **Is the production strain genetically modified?** Yes, go to 2.

2. **Is the production strain modified using rDNA techniques?** Yes. The donor of this G4-amylase gene is *Pseudomonas stuzteri* and the host is *Bacillus licheniformis*. Go to 3a.

3a. **Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food?** Yes. Three previous generations of the enzyme, produced in *Bacillus subtilis*, have been determined to be GRAS. Furthermore, amylases with the designation 3.2.1.1, which are produced from fungal, bacterial and cereal sources, are widely used in the food industry and have been so for decades. Maltotetraohydrolases (EC 3.2.1.60) from *Pseudomonas stutzeri* strains, which are closely related to the three generations of this G4-amylase, have been used for producing maltotetraose and malto-tetraose syrups for use in foods since the 1980s[25, 32,-33]. Maltotetraose-forming amylase from *P. stutzeri* is listed as a natural additive in the Korean Food Additives Code. Go to 3c.

3c. **Is the test article free of transferable antibiotic resistance gene DNA?** Yes. Transformable DNA was not detected at or above the limit of 5 ng/ml in the enzyme preparation manufactured using this host and production process. Go to 3e.

3e. **Is all other introduced DNA well characterised and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?** Yes. The only DNA introduced is the endogenous *B. licheniformis* cat gene and this G4-amylase gene. The inserted sequences do not code for any known toxins. Go to 4.

4. **Is the introduced DNA randomly integrated into the chromosome?** No. The construct is designed to integrate into the *cat* locus by Campbell type recombination Go to 5.

5. **Is the production strain sufficiently well characterised so that one may reasonably conclude that unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed?** Yes. Given the targeted integration (see 4 above) there is no concern for pleiotropic effects. Go to 6.

6. **Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?** Yes. The *B. licheniformis* host and methods of integration of the enzyme gene have been used by Genencor for production of many enzyme production organisms. Toxicology testing on 5 products from this strain lineage confirms the safety of the lineage. **Accept.**

4

Appendix 4 – GRAS Panel Report

Michael W. Pariza Consulting, LLC
7102 Valhalla Trail
Madison, WI 53719

Michael W. Pariza, Member

March 7, 2008

Alice J. Caddow
Vice President, Regulatory
And Environmental Affairs
Genencor, A Danisco Division
925 Page Mill Road,
Palo Alto, CA 94304

Dear Ms. Caddow:

I have reviewed the information you provided on Danisco's amylase SAS3 enzyme preparation expressed in a genetically modified strain of *Bacillus licheniformis* Bra 7. In this evaluation, I considered the biology and the safe lineage of the genetically-modified *Bacillus licheniformis* Bra 7 production organism, information provided by Danisco on the safety of the modified gene for amylase SAS3 and its expression product (the amylase SAS3 protein), and information regarding the gene source organism which has been reclassified as *Pseudomonas stutzeri* IAM1504 based on 16s rDNA sequence analysis (it had been previously misclassified as *P. saccharophilia* IAM1504 based on older biochemical/physiological assessment methodology).

Amylase enzyme preparations designated D34 and SAS2 that are substantially equivalent to SAS3 were previously determined to be GRAS by scientific procedures, as was SAS3 expressed in *B. subtilis*. The genes for D34 and SAS2 were both derived from *Pseudomonas stutzeri* IAM1504 and cloned into a strain of *Bacillus subtilis* that has a long history of safe use in food enzyme manufacture. SAS3 differs from the SAS2, D34, and *Pseudomonas stutzeri* IAM1504 wild-type amylase (designated PS4wt) catalytic core, by the number of amino acid modifications introduced into the amylase gene. These mutations were introduced to enhance stability and catalytic activity, thereby improving enzyme performance in baking applications.

The *B. licheniformis* Bra 7 production organism, which has been genetically modified to

express the amylase SAS3 gene, has a long history of safe use in food enzyme manufacture. It was derived from a *B. licheniformis* safe strain lineage that has been previously described in GRAS assessment documents. These documents were reviewed by expert scientific panels, and determined to be GRAS by scientific procedures for food enzyme manufacture.

Based on these considerations, I conclude that the Danisco amylase SAS3 enzyme preparation derived from a genetically modified strain of *Bacillus licheniformis* Bra 7 by the manufacturing procedure you have described is GRAS for use in baking applications under the intended conditions of use that you have described.

Please note that this is a professional opinion directed at safety considerations only and not an endorsement, warranty, or recommendation regarding the possible use of the subject product by you or others.

Sincerely,

(b)(6)

Michael W. Pariza
Wisconsin Distinguished Professor of
Food Microbiology and Toxicology
Member, Michael W. Pariza Consulting LLC

RECEIVED
MAR 18 2009
BY: (b)(6)

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March 17, 2009

Dr. Robert Martin
Office of Food Additive Safety, HFS-255
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

RE: GRN 000277
Dear Dr. Martin:

At the request of the reviewers of this GRAS Notice, I am writing to explain the relationship of amylases D34 and SAS2 to SAS3 G4-amylase (modified maltotetraohydrolase). These two amylases are referenced in the GRAS determination letter from Dr. Michael W. Pariza, March 7, 2008.

D34 and SAS2 amylases are precursors to SAS3. SAS3 is referred to as G4-amylase in GRAS notice 000277. D34 and SAS2 are protein engineered versions of the G4 maltotetraohydrolase from *P. stutzeri* expressed in *B. subtilis*. They are not as effective in anti-staling properties as SAS3, so are being replaced by SAS3. Danisco assessed the safety of both of these enzyme preparations. An expert panel of Dr. Michael Pariza, Dr. Herbert Blumenthal and Dr. Joseph Borzelleca reviewed the GRAS determination information for both enzyme preparations and concluded that the enzyme preparations are GRAS for use in food as described by Danisco, i.e., to retard the staling of bread and other baked goods. I have enclosed copies of the panel GRAS letters for both enzymes.

If you have questions or require additional information, please contact me at 650-846-7557 or fax at 650-845-6505.

Sincerely,

(b)(6)

Alice J. Caddow
Vice President of Regulatory
And Environmental Affairs

Enclosures

Evaluation of the GRAS Status of a GM-derived Amylase Enzyme for Foods

Summary Report, 27 May 2004

Introduction

An amylase enzyme produced by fermentation of a genetically modified (GM) strain of *Bacillus subtilis* has been developed for use in food. This enzyme is designated as maltotetraohydrolase PS4wt, SAS Gen1 Amylase or Amylase D34.

A Panel of expert scientists (the Panel) qualified by scientific and biomedical training and national and international experience to evaluate the safety of foods and food ingredients was assembled by Danisco USA to assess the potential GRAS status of this product. The resumes of the Panel appear in section VI. The Panel was provided with:

- a search of the scientific literature conducted by Danisco via the MEDLINE, Chemtox and Chem Abstracts databases through March 2004;
- dietary exposure data:
- a detailed description of the GM organism and the isolation, modification and characterization of the amylase gene; and
- reports of safety studies and relevant FAO/WHO JECFA review documents on other enzymes for food use.

The Panel independently critically evaluated these documents and other materials deemed relevant and conferred by telephone. They then met with representatives of Danisco USA and Genencor International at Danisco facilities in Ardsley, NY, on May 27, 2004, and considered all available information. The Panel then met in executive session and developed the conclusion presented below.

Description, use and production

Amylase D34 is an enzyme that catalyses the hydrolysis of 1,4-α-D-glycosidic linkages in amylaceous polysaccharides so as to remove successive maltotetraose residues from the non-reducing chain ends. The effect of this is to delay the staling process in bread and other baked goods and thereby extend the period in which the products have acceptable organoleptic properties. This is a benefit to bread producers and consumers as it will extend shelf life. The ability of Amylase D34 to form maltotetraose can also be exploited in the starch processing industry when producing maltotetraose.

The organism from which the gene was derived, *Pseudomonas saccharophila* IAM1504 was reclassified as *P. stutzeri*. This organism does not have a long history of safe use in food. Accordingly, its appropriateness as a gene donor was based on an extensive search of the scientific literature. Several isolated references were found suggesting a link between *P. stutzeri* exposure and human infections. In most cases, however, a causative link was not confirmed or the effects were traced to strains containing specific genes not present in the strain used in this work.

The host organism *B subtilis* BG3594 Δ amy was developed by Genencor using a specific procedure with the non-toxigenic *B. subtilis* 168, which has a long history of safe use as a host for production of food enzymes by fermentation.

The gene encoding amylase D34 was prepared by successive cycles of random, site specific and site scanning mutagenesis. This gene was inserted using recombinant techniques into the host organism *Bacillus subtilis* BG 3594 Δ amy. The modified enzyme has significantly improved thermostability and exo-specificity. The gene, the vector and the host organism were fully characterized.

The construction of the vector was documented in detail and the genetic stability of the recombinant organism were assessed and judged to be appropriate for the production of food grade materials.

Amylase D34 is produced during aerobic fermentation in a straight or fed-batch process and secreted into the medium. The enzyme is recovered by lysing the cells with lysozyme to facilitate flocculation, filtration or centrifugation, and concentration via ultrafiltration.

Estimated exposure

The typical use level of amylase D34 is 0.5-4.0 mg enzyme protein/kg flour, where one gram of enzyme protein corresponds to 45,000 DAU (Danisco amylase units). Thus the typical amylase use level would be 22.5-180 DAU/kg flour. The enzyme will be denatured during heat processing of foods (baking, pasteurization).

Consumption of relevant foods was determined from USDA's 1996 Continuing Survey of Food Intakes by Individuals (CSFII 1996), the 1995 Danish Food Survey (DFS 1995) and Euromonitor. The Italian intake values from the Euromonitor were used in the analysis as they represented the highest average intake. Considering this value, the range of enzyme use levels corresponds to intakes of 0.07 - 0.59 DAU/kg body weight/day or 1.6 - 13.0 µg enzyme protein/kg bw/day.

Safety

A toxicological test program was undertaken using preparations of amylase D34. The testing included acute and subchronic feeding studies as well as tests for mutagenesis.

- **Acute Oral Toxicity Study in the Rat**– No signs of toxicity were seen in an acute study at the dose of 2000 mg total protein (111 mg enzyme protein)/kg bw, equivalent to 5000 DAU/kg body weight.

- **13-week Feeding Study in the Rat** - Amylase D34 given daily by gavage to rats for 13 weeks at dosages of 4.3, 8.7 and 17.5 mg enzyme protein/kg body weight/day (=194, 392 and 788 DAU/kg bw/day) respectively, caused no signs of toxicity. The no-observed-adverse-effect-level (NOAEL) was thus 788 DAU/kg bw/day or 17.5 mg enzyme protein/kg bw/day. This value is 1350 times the upper level estimated intake.

- **Ames Test**– The enzyme was non-mutagenic in tests with 5 strains of *Salmonella typhimurium* with/without mammalian liver enzyme metabolic activation.

- ***In vitro* Mammalian Chromosomal Aberration Study with Human Lymphocytes** - The enzyme was non-clastogenic in studies with/without mammalian liver en-zyme metabolic activation.

The safety of the amylase was further assessed according to the decision tree in the Pariza-Johnson guidelines for assuring the safety of new enzymes. The conclusion was that the enzyme is "Accepted" as safe for its intended use.

Danisco and Genencor informed the Panel that the safety studies will be published shortly.

Conclusion

The Panel concluded, following its individual and collective critical evaluation of the data and information summarized above, that amylase meeting the specifications described herein and produced in compliance with cGMP is Generally Recognized as Safe (GRAS) by scientific procedures for use in foods as described in the attached documentation.

(b)(6)

Herbert Blumenthal, Ph. D.
Consultant

(b)(6) 27 May 2004

Michael W. Pariza, Ph. D.
Director, Food research Institute
Chair, Dept. of Food Microbiology and Toxicology
Wisconsin Distinguished Professor
University of Wisconsin - Madison

(b)(6) 27 May 2004

Joseph F. Borzelleca, Ph. D.
Professor of Pharmacology and Toxicology
Medical College of Virginia

Evaluation of the GRAS Status of a GM-derived Amylase Enzyme for Foods

Summary Report, 17 October 2005

Introduction

An amylase enzyme produced by fermentation of a genetically modified (GM) strain of *Bacillus subtilis* has been developed for use in food. This enzyme is designated as 1,4-α-D-Glucan malto-tetraohydrolase or Amylase SAS2. It is substantially equivalent to the enzyme Amylase D34 (also referred to as Amylase SAS1) which was previously determined to be GRAS by an expert scienti-fic panel. The donor organism for the enzyme gene and the host organism in which the gene is inserted are the same for Amylase D34 and Amylase SAS2.

A Panel of expert scientists (the Panel) qualified by scientific and biomedical training and national and international experience to evaluate the safety of foods and food ingredients was assembled by Danisco USA to assess the potential GRAS status of this product. The resumes of the Panel appear in Section VI of the complete dossier. The Panel was provided with:

- a search of the scientific literature conducted by Danisco via the MEDLINE, Chemtox and Chem Abstracts databases through August 2005;
- dietary exposure data:
- a detailed description of the GM organism and the isolation, modification and characterization of the amylase gene; and
- reports of safety studies and relevant FAO/WHO JECFA review documents on other enzymes for food use.

The Panel independently critically evaluated these documents and other materials deemed relevant and conferred by telephone. They then spoke with representatives of Danisco USA and Genencor International via teleconference on October 17, 2005, and considered all available information. The Panel then met in executive session and developed the conclusion presented below.

Description, use and production

Amylase SAS2 is an enzyme that catalyses the hydrolysis of 1,4-α-D-glycosidic linkages in amyl-aceous polysaccharides so as to remove successive maltotetraose residues from the non-reducing chain ends. The effect of this is to delay the staling process in bread and other baked goods and thereby extend the period in which the products have acceptable organoleptic properties. This is a benefit to bread producers and consumers as it will extend shelf life.

Amylase SAS2 is a variant of the wild type maltotetraohydrolase (PS4wt) from *Pseudomonas sac-charophila* strain IAM 1504. The wild type enzyme PS4wt encoded by the gene mta of *P. sac-charophila* strain IAM 1504 was modified as follows: The starch-binding domain was removed and 17 out of the remaining 429 amino acids of the catalytic core were changed in order to im-prove thermostability and exo-specificity of the enzyme. Furthermore, one methionine residue has been added at the N-terminus of the mature enzyme because that improved fermentation yields. Compared to Amylase D34, 8 additional mutations were introduced to generate Amylase SAS2.

The half-life of Amylase D34 was increased more than 50 fold from 1-2 min. at 60°C (140°F) to about 100 min. at 60°C (140°F) as compared to the wild type PS4wt. The 8 additional muta-tions further extended the half-life of Amylase SAS2 an additional 7-8 times at 75 and 80°C (167 and 176°F) and 3 times at 85°C (185°F) as compared to Amylase D34.

The gene encoding amylase SAS2 was prepared by successive cycles of random, site specific and site scanning mutagenesis combined with screening for thermostability and exo-specificity. This gene was

inserted using recombinant techniques into the host organism *Bacillus subtilis* BG 3594-3, which was developed by Genencor using a specific procedure with the non-toxigenic *B. subtilis* BG125. The latter was derived from *B. subtilis* 168, which has a long history of safe use as a host for production of food enzymes by fermentation, thereby demonstrating a safe strain lineage through the SAS2 production host organism.

The gene, the vector and the host organism were fully characterized. The construction of the vector was documented in detail and the genetic stability of the recombinant organism was as-sessed and judged to be appropriate for the production of food grade materials.

Amylase SAS2 is secreted into the medium during aerobic fermentation in a straight or fed-batch process. The enzyme is recovered by flocculation, filtration or centrifugation, and concentration via ultrafiltration and precipitation.

Estimated exposure

The typical use level of Amylase SAS2 is 0.5-15.0 mg enzyme protein/kg flour, where one milli-gram of enzyme protein corresponds to 8.1 BMK units (see assay section, Appendix 4). Thus the typical amylase use level would be 4-120 BMK/kg flour. The enzyme will be denatured during heat processing of foods (baking, pasteurization).

Consumption of relevant foods was determined by consulting USDA's 1996 Continuing Survey of Food Intakes by Individuals (CSFII 1996), the 1995 Danish Food Survey (DFS 1995) and Euromo-nitor. The Italian intake values from the Euromonitor were used in the analysis as they represent-ed the highest average intake. Considering this value, the range of enzyme use levels corres-ponds to intakes of 0.01 - 0.39 BMK/kg bw/day or 1.6 - 49.0 μg enzyme protein/kg bw/day.

Safety

The safety of Amylase SAS2 as a food processing aid in bread was assessed based on the sub-stantial equivalence of Amylase D34 and Amylase SAS2 enzymes in terms of identity, function-ality, chromatographic properties, nearly identical amino acid sequence and the construction and production processes. Given this equivalence, the safety studies conducted on Amylase D34 were deemed to be applicable. These included

- Acute Oral Toxicity Study in the Rat
- 13-week Feeding Study in the Rat
- Ames Test
- *In vitro* Mammalian Chromosomal Aberration Study with Human Lymphocytes

No additional toxicological tests were deemed necessary on preparations of Amylase SAS2. How-ever, it was also noted that:

- Sequence homology to known enterotoxins was investigated and none was found.
- SAS2 was evaluated via the Pariza-Johnson decision tree for safety of microbial enzymes and was "Accepted" as safe for its intended use.
- The enzyme will be denatured during baking prior to ingestion, so dietary exposure to active enzyme will be negligible.
- It was concluded that no toxic peptides should be formed during passage through the gastroin-testinal tract.
- Thousands of proteins are consumed daily by individuals and only a few of these are potential-ly toxic. Thus it is virtually impossible to imagine that the change of 8 amino acids would sig-nificantly alter the safety of the enzyme.

In all aspects considered, it was found that the safety of Amylase SAS2 did not differ from that of Amylase D34. The NOAEL determined from the 13 week study of Amylase D34 is 17.5 mg enzyme protein/kg bw/day. The worst-case scenario of daily bread consumption is 0.049 mg enzyme protein/kg bw/day. Thus the margin of safety is 357-fold.

Conclusion

The Panel concluded, following its individual and collective critical evaluation of the data and information summarized above, that amylase meeting the specifications described herein and produced in compliance with cGMP is Generally Recognized as Safe (GRAS) by scientific procedures for use in foods as described in the attached documentation.

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(b)(6)

17 October 2005

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