ORIGINAL SUBMISSION

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4 June, 1999

Linda S. Kahl, Ph.D.
Regulatory Policy Branch, HFS-206
Center for Food Safety and Applied Nutrition
Food and Drug Administration
200 C Street, S.W.
Washington, D.C. 20204

Dear Dr. Kahl,

We are hereby submitting, in triplicate, a generally recognized as safe (GRAS) notification, in accordance with proposed 21 C.F.R. § 170.36, for Novo Nordisk's α-amylase enzyme preparation, produced by *Bacillus licheniformis* carrying the gene encoding a modified α-amylase from *Bacillus stearothermophilus*. The α-amylase enzyme preparation is to be used in the food industry as a processing aid in the liquefaction of starch.

Please contact me by direct telephone at 919 494-3152 or direct fax at 919 494-3420 if you have any questions or require additional information.

Sincerely,

Scott H. Shore, Ph.D.
Senior Regulatory Specialist

Enclosures (3 binders)

1999 JUN -7 P 3:40

3 June, 1999

Office of Premarket Approval
Center for Food Safety and Applied Nutrition
Food and Drug Administration
200 C Street, S.W.
Washington, D.C. 20204

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RE: GRAS Notification - Exemption Claim

Pursuant to the proposed 21C.F.R. §170.36(c)(1) Novo Nordisk BioChem North America Inc. hereby claims that an α-amylase enzyme preparation produced by submerged fermentation of *Bacillus licheniformis* carrying the gene encoding a modified α-amylase from *Bacillus stearothermophilus* is Generally Recognized as Safe; therefore, it is exempt from statutory premarket approval requirements.

The following information is provided in accordance with the proposed regulation:

Proposed §170.36(c)(1)(i) *The name and address of the notifier.*

Novo Nordisk BioChem North America Inc.
77 Perry Chapel Church Rd., Box 576
Franklinton, NC 27525

Proposed §170.36(c)(1)(ii) *The common or usual name of notified substance.*

α-Amylase enzyme preparation from *Bacillus licheniformis* carrying the gene coding for a modified α-amylase from *Bacillus stearothermophilus*.

Proposed §170.36(c)(1)(iii) *Applicable conditions of use.*

The above described α-amylase preparation is to be used in the food industry as a processing aid in the liquefaction of starch. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following Good Manufacturing Practices.

Proposed §170.36(c)(1)(iv) *Basis for GRAS determination.*

This GRAS determination is based on scientific procedures.

Proposed §170.36(c)(1)(v) *Availability of information.*

A notification package providing a summary of the information which supports this GRAS determination is enclosed with this letter. 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.

3 June, 1999
Date

John Carroll
Director, Regulatory Affairs

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Termamyl® SC

an α-amylase preparation

produced by *Bacillus licheniformis* expressing

a gene encoding a modified

α-amylase from *Bacillus stearothermophilus*

Kim Rygaard Nielsen, Enzyme Regulatory Affairs, NNA/S, Denmark
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1. GENERAL INTRODUCTION

Termamyl® SC is the Novo Nordisk A/S trade name used for an α-amylase preparation produced by submerged fermentation of *Bacillus licheniformis* carrying a modified gene coding for α-amylase from *Bacillus stearothermophilus*.

The enzyme preparation is to be used in the food industry as a processing aid in the liquefaction of starch.

The enzyme is a heat-stable α-amylase, which can operate at lower pH and lower calcium levels than conventional thermostable α-amylases. The enzyme is an endo-amylase, which hydrolyses 1,4-α-glucosidic linkages in amylose and amylopectin. Starch is thereby broken down to soluble dextrins and oligosaccharides.

The following sections provide information on the enzyme and the production organism which are the basis for our determination of the general recognition of safety of an α-amylase enzyme preparation (commercial product trade name, Termamyl SC) produced by *B. licheniformis* expressing a gene encoding a modified α-amylase from *B. stearothermophilus*. Our safety evaluation in Section 7 includes an evaluation of the published literature supporting the safety of the production strain, the enzyme, and the manufacturing process as well as an evaluation of potential dietary exposure to the enzyme preparation.

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food[1]. The production organism for Termamyl SC, *B. licheniformis* expressing a modified α-amylase from *B. stearothermophilus*, is discussed in sections 2 and 7. Carbohydrase and protease enzyme products from *B. licheniformis* have been affirmed by FDA as GRAS[2]. In addition, *B. stearothermophilus* α-amylase has been affirmed by FDA as GRAS[3] and FDA has published a notice of filing of a petition for affirmation of GRAS status for an α-amylase enzyme preparation derived from *B. licheniformis* expressing a *B. stearothermophilus* α-amylase[4].

An essential aspect of the safety evaluation of food components derived from genetically modified organisms is the identification and characterization of the inserted genetic material[5-10]. The genetic modifications used to develop the production microorganism and α-amylase enzyme are well defined and are described in Section 2.

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Data showing this modified α-amylase to be substantially equivalent[8-13] to naturally occurring *B. stearothermophilus* and other α-amylases is provided in Section 3.

The safety evaluation of the processing aids and other substances used in the manufacturing process is included in Section 4. The composition and specifications of the commercial product, Termamyl SC, are given in Section 5. Section 6 describes the application of Termamyl SC including use levels and an assessment of enzyme residues in the final food. Finally, an evaluation of potential dietary exposure is included as part of the safety evaluation in Section 7.

It should be noted that within some of the attachments, the terms "SP 961", and "Low Ca BS Amylase" are used to describe Termamyl SC. These two names are internal names used before a decision concerning the trade name was made.

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The microbial production strain for Termamyl SC, designated LiH 1464, (production code Bc-1 (NN049406)) is a derivative of the *B. licheniformis* natural isolate, American Type Culture Collection Strain (ATCC) 9789. This ATCC 9789 strain is the ancestor of *B. licheniformis* strains that have been used safely for industrial production of enzymes since 1972 including an enzyme preparation that was the subject of the GRAS affirmation petition[14] submitted by Novo Nordisk and affirmed by FDA in 1983[15]. The classification of this strain and the production microorganism is based on generally accepted taxonomic characteristics[16] and the identity of the production strain was confirmed by 16S rRNA sequence analysis[17].

Strain LiH 1464 was constructed by common transformation procedures using well-known plasmid vectors[18,19] and well-characterized *B. licheniformis* and *B. stearothermophilus* α-amylase DNA sequences[20,21]. The recombinant plasmid DNA was integrated into the *B. licheniformis* host strain chromosome by homologous recombination. The *B. stearothermophilus* α-amylase gene (amyS) was modified by well-known techniques designed to introduce specific desired changes in the coding sequence[22]. The identity and location of these changes were verified by DNA sequencing in both orientations several times. The genetic construction was evaluated at every step to assess the incorporation of the desired functional

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genetic information and to ensure that no unintended sequences were incorporated in the production strain.

This genetically modified production organism complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms[23]. The genetically modified strain meets the criteria for a safe production microorganism as described by Pariza and Foster and several expert groups[5-10]. These criteria include the identification and characterization of the host strain, plasmid vector, and inserted genetic sequences, all described below.

2.2 Recipient Organism

The recipient microorganism, designated SJ1707, used in the construction of the α-amylase production strain is a sporulation deficient and alkaline protease negative derivative of a natural isolate of *B. licheniformis*, ATCC 9789.

2.3 Plasmid

The 4.44 kb amylase expression plasmid, pLiH1346, used in the construction of the production strain, enabled incorporation and amplification of the modified *B. stearothermophilus* α-amylase gene in the recipient organism. Plasmid pLiH1346 contains strictly defined bacterial chromosomal DNA fragments, DNA from well-characterized bacterial cloning vectors, and specific synthetic oligonucleotide sequences. The particular DNA sequences include: coding sequence for the modified α-amylase enzyme; 5' flanking fragments from the wild-type *B. licheniformis* α-amylase coding sequence, 3' flanking fragments from the wild-type *B. stearothermophilus* α-amylase coding sequence, a non-functional origin of replication from plasmid pE194, and well-known sequences from *Bacillus* cloning vector plasmid pUB110. Plasmids pE194 and pUB110 are certified plasmid vectors under the NIH Guidelines[24].

Plasmid pLiH1346 contains the following genetic material (see Appendix A):

1.80 kb DNA from the *Bacillus* cloning vector, pUB110[18]
0.28 kb DNA from pE194 containing nonfunctional replication origin[19]
0.61 kb DNA from 5' region of the *B. licheniformis* α-amylase gene[20]
1.75 kb DNA from the coding sequence and 3' region of the modified *B. stearothermophilus* α-amylase gene[21,Section 3.2]

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2.4 Stability of the Transformed Plasmid Genetic Sequences

The presence and configuration of the introduced DNA sequences was determined by Southern hybridization to assess the stability and potential for transfer of genetic material as a component of the safety evaluation of the production microorganism[5-10]. The transforming plasmid DNA sequences are stably integrated into the *B. licheniformis* chromosome and do not contain any sequences known to promote gene transfer. The introduced genetic material is poorly mobilizable for genetic transfer to other organisms and is mitotically stable[22].

2.5 Absence of Transformable rDNA in the Enzyme Preparation

The absence of transformable recombinant DNA sequences used in the production strain construction is a component of the safety evaluation of the production microorganism[6,7]. A sample of the Termamyl SC enzyme preparation was analyzed for the presence of transformable DNA sequences[25]. The analysis was based on selection of kanamycin resistant transformants. No transforming DNA capability was detected in the sample of the Termamyl SC enzyme preparation analyzed. Therefore, there is no potential for transfer of recombinant DNA sequences to other organisms which ingest foods processed with the α-amylase enzyme preparation.

2.6 Absence of Production Organism in the Product

The absence of the production organism is an established specification for the commercial product, Termamyl LC. The production organism does not end up in food and therefore the first step in the safety assessment as described by IFBC[6] is satisfactorily addressed.

2.7 Antibiotic Resistance Gene

Plasmid pLiH 1346 contains a kanamycin resistance gene, encoding an aminoglycoside 3'-phosphotransferase II protein, APH(3') II. FDA has evaluated the safety of this gene and gene product for use in the development of genetically engineered tomato, oilseed rape, and cotton[26]. The FDA's evaluation of issues and the agency's conclusions are also applicable for assessment of the kanamycin resistance gene present in the Termamyl SC production strain. As discussed by FDA in its decision[26] and further elaborated in the draft guidance and report on the use of antibiotic resistance marker genes in transgenic plants[27], the evaluation of the safety of the use of an antibiotic resistance marker gene should include an assessment of

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the safety of the protein encoded by the gene and consideration of the potential for horizontal transfer of the antibiotic resistance gene.

As stated above in Section 2.5, no transformable recombinant DNA sequences were detected in the Termamyl SC enzyme product and as stated in Section 2.6, the absence of the production organism is an established specification for the Termamyl SC enzyme product. Consequently, the kanamycin antibiotic resistance gene would not be present in either the Termamyl SC enzyme product or in foods derived from starch processed with the Termamyl SC enzyme product. Therefore, the use of the Termamyl SC enzyme product as a processing aid in the liquefaction of starch does not pose a safety concern due to any potential for transfer of the kanamycin resistance gene to other organisms.

FDA's safety evaluation and conclusions regarding the potential direct effect of ingestion of the APH(3') II protein and the potential effect of the biological activity of APH(3') II protein on the therapeutic efficacy of orally administered antibiotics[26] is also applicable to the use of the kanamycin resistance gene in the Termamyl SC production strain. The kanamycin antibiotic resistance gene used in the Termamyl SC production strain is the same as the kanamycin resistance gene evaluated by FDA[26] and in each case encodes the same protein, APH(3') II. High temperature, in excess of 100° C, as well as other conditions and steps used during industrial starch processing (low pH, filtration, ion exchange)[28-31] would be expected to inactivate and remove APH(3') II protein. It is unlikely there would be any APH(3') II protein in any final food product derived from starch processed with the Termamyl SC enzyme product. In addition, the biological activity of APH(3') II is destroyed during digestion. Therefore, the use of the kanamycin resistance gene encoding APH(3") II in the Termamyl SC production strain will not compromise the therapeutic use of orally administered kanamycin or neomycin.

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3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1 Enzyme Identity

Key characteristics of Termamyl SC enzyme are given below. It is an α-amylase as defined by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology[32].

Classification:	α-amylase (generic name)
IUB EC number:	3.2.1.1
Systematic name:	1,4-α-D-Glucan glucanohydrolase
CAS No.:	9000-90-2
EINECS No.:	232-565-6
Specificity:	1,4-α-glucosidic linkages in amylose and amylopectin.
Molecular weight:	55 kDa
Amino acid sequence:	The total nucleotide and amino acid sequences have been determined

3.2 Amino Acid Sequence (see Appendix B)

The Termamyl SC α-amylase enzyme has been modified to improve enzyme performance by increasing stability at low calcium concentration and low pH. The starting point for the Termamyl SC enzyme was the naturally occurring *B. stearothermophilus* α-amylase gene[33] used in the production of Novo Nordisk's α-amylase enzyme preparation (BSG amylase) derived from a strain of *B. licheniformis* expressing the *B. stearothermophilus* α-amylase gene[33,34]. The modified Termamyl SC enzyme has a deletion of two amino acids (positions 181 and 182) and a single substitution (position 193) as compared to the original *B. stearothermophilus* α-amylase.

Naturally occurring α-amylase enzymes require calcium ions for stability. In industrial applications, such as starch processing, up to 40 ppm calcium is added. The addition of calcium is costly and the calcium must be removed after the starch liquefaction step to avoid fouling of equipment and inactivation of glucose isomerase. This is done by ion exchange which results in large volumes of waste water with high levels of salts.

Several concepts were applied in selecting the appropriate amino acid residues to be modified in order to achieve the desired effect. The identification of specific residues to be changed was based on information from published scientific literature on the structure and

function of α-amylases, analysis of publicly accessible protein sequence databases, and internal Novo Nordisk research.

The general structure of α-amylases is characterized by interactions of three common domains and several calcium ion binding sites[35,36]. The primary approach used to develop an improved α-amylase was to change amino acid residues that would result in more stable domain/domain and calcium ion interactions.

The complete amino acid sequence (using single letter amino acid code) for the Termamyl SC mature enzyme is given as the top line of the sequence alignment shown in Appendix B. The amino acid residue designated 1 in this figure is the first alanine in the mature Termamyl SC α-amylase enzyme sequence.

Five other *B. stearothermophilus* α-amylase amino acid sequences are aligned and compared with the Termamyl SC amino acid sequence in Appendix B. The sequences are designated by their National Center for Biotechnology Information (NCBI) Entrez Protein Sequence Database locus or accession designations (http://www.ncbi.nlm.nih.gov/Entrez/protein.html). The five other *B. stearothermophilus* α-amylase amino acid sequences shown in Appendix B are: A5451[33], A24549[37,38], A24436[39], AAB86961[40-43], and AAA22227[44].

A change between the Termamyl SC enzyme and any of the other five *B. stearothermophilus* α-amylase enzymes is indicated by a red letter in the other *B. stearothermophilus* sequence and a red asterisk above the Termamyl SC sequence. The three differences between the Termamyl SC α-amylase and the original *B. stearothermophilus* α-amylase, A5451, are indicated above the Termamyl SC sequence by blue arrows ↓.

Two amino acids were deleted in the Termamyl SC enzyme, isoleucine at position 181 and glycine at position 182. A single amino acid change was incorporated at position 193, substituting a phenylalanine in Termamyl SC for the asparagine in the original *B. stearothermophilus* α-amylase. These changes are indicated in Appendix B by the locations of the arrows. These changes provide increased stability at low calcium concentration and low pH.

3.3 Sequence Comparison to *B. stearothermophilus* α-amylases (see Appendix B)

The Termamyl SC α-amylase amino acid sequence has been compared to other published and available *B. stearothermophilus*

α-amylase amino acid sequences[33,37-44] (Appendix B). Amino acid identity among these *B. stearothermophilus* α-amylases is indicated in the table in Appendix C.

Termamyl SC is 99.4% identical to the original *B. stearothermophilus* α-amylase[33], A5451, and ranges between 96.5% to 99.0% identical to the other *B. stearothermophilus* α-amylases. This is within the range of similarity of the other *B. stearothermophilus* α-amylases with each other as shown in the table.

None of the differences among these α-amylases are found in conserved amino acid residues identified as structurally or functionally important for α-amylases in general[35,36].

3.4 Enzymatic Activity

Termamyl SC α-amylase is functionally equivalent to another α-amylase from *B. stearothermophilus*, designated Termamyl S, which corresponds to the α-amylase that is the subject of GRASP 0G0363[34] and corresponds to the amino acid sequence A5451[33] in Appendix B. Two experiments (Appendices D and E) were carried out to verify that Termamyl SC α-amylase exhibited catalytic properties and functional effects in starch hydrolysis substantially similar another α-amylase from *B. stearothermophilus*. The experiments conducted were: monitoring changes in carbohydrate spectra during starch hydrolysis by HPLC (Appendix D) and analysis of glucose yield and oligosaccharide composition by HPLC (Appendix E). These experiments demonstrate that Termamyl SC is functionally equivalent to Termamyl S, an α-amylase derived from a unmodified, naturally occurring *B. stearothermophilus* gene, with respect to the starch hydrolytic process and products.

4. MANUFACTURING PROCESS

This section describes the manufacturing process for Termamyl SC. The quality management system used in the manufacturing process for Termamyl SC complies with the requirements of ISO 9002. Termamyl SC is also manufactured in accordance with current good manufacturing practices.

4.1 Raw Materials

Raw materials used for fermentation and for recovery conform to Food Chemicals Codex[45] (FCC) specifications except those raw materials which do not appear in the FCC. For those not appearing

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in the FCC, internal specifications have been made in line with FCC requirements. On arrival at Novo Nordisk A/S, the raw materials are sampled by the Quality Control Department and subjected to the appropriate analyses to ensure their conformance to specifications.

The antifoams and flocculants used in fermentation and recovery and listed below in Sections 4.2.1 and 4.3.1 are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 10, 1998. The maximum use level of these antifoams and flocculants in the Termamyl SC product is less than 1%.

4.2 Fermentation Process

Termamyl SC is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *B. licheniformis* described in Section 2. A flow sheet of the fermentation process is shown in Appendix F, Figure 1. 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 to ensure absence of foreign microorganisms and to confirm strain identity.

4.2.1 Raw Materials for the Fermentation Process

The raw materials used in the fermentation process are listed below. The list includes both the raw materials used for the seed fermentor as well as those used for the main fermentor.

- Starch and/or starch hydrolysates*, Glucose*
- Potato Protein
- Termamyl (α-amylase)
- Corn Steep Liquor, Soy (Soy Bean Meal or Soy Grits)
- Minerals (phosphates, sulfates)*
- Alkali and acid (such as ammonia, sodium or potassium hydroxide, sodium carbonate and phosphoric acid)* for pH adjustments
- Polymeric antifoam compounds- P2000 (polypropylene glycol)* and Pluronic PE 6100 (polyoxypropylene-polyoxyethylene copolymer)
- Potable water

*Conform to FCC specifications

4.2.2 Production Organism

Each batch of the fermentation process is initiated with a lyophilized stock culture of the *B. licheniformis* production strain, production code Bc-1, described in Section 2. Each new batch of the stock

culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

4.2.3 Criteria for the Rejection of Fermentation Batches

Growth characteristics during fermentation are observed both macroscopically and microscopically. Samples are taken from both the seed fermentor and the main fermentor before inoculation, at regular intervals during cultivation, and before transfer/harvest. These samples are tested for microbiological contamination by microscopy and by plating on a nutrient agar followed by a 24-48 hour incubation period.

The fermentation is declared "contaminated" if one of the following conditions are fulfilled:

1. Infection is observed in 2 or more samples by microscopy
2. Infection is observed in two successive agar plates at a minimum interval of 6 hours

Any contaminated fermentation is rejected.

4.3 Recovery Process

The recovery process is a multistep operation which starts immediately after the fermentation process and consists of both the purification and the formulation processes. A flow sheet of the recovery process is shown in Appendix F, Figure 2.

4.3.1 Raw Materials in the Recovery Process

- Alkali and acids for pH adjustment (sodium or potassium hydroxide, acetic acid, phosphoric acid)*
- Precipitation/flocculating agent [calcium chloride*, sodium aluminate, Superfloc A130 (polyacrylamide acrylate copolymer) and C521 (Dimethylamine epichlorohydrin copolymer)]
- Filter aids (diatomaceous earth/Celite, Dicalite, Perlite or similar)*
- Stabilizers and formulation aids (sodium chloride, sucrose)*
- Water

*Conform to FCC specification

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4.3.2 Purification Process

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

1. Pretreatment - flocculation/pH adjustment
2. Primary Separation - drum filtration
3. Concentration - ultra filtration and evaporation
4. Germ Filtration

Pre- and Germ Filtration are performed for removal of residual production strain organisms and as a general precaution against microbial degradation.

4.3.3 Formulation and Standardization Processes

The enzyme concentrate is blended with water, sodium chloride, and sucrose and, if necessary, adjusted to the desired pH. The product is standardized according to the product specification.

4.4 Quality Control of Finished Product

The final products are analyzed according to the specifications given in Section 5.

5. COMPOSITION AND SPECIFICATIONS

5.1 Formulation

Termamyl SC will be available in a liquid formulation with an activity of 120 KNU(S)/g and currently with the following typical composition:

Enzyme solids (E-TOS)	approx. 2%
Sodium Chloride	approx. 14%
Sucrose	approx. 30%
Water	approx. 54%

Further information on the product is given in the enclosed Product Sheet (Appendix G).

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5.2 Specifications

Termamyl SC complies with the purity criteria recommended for enzyme preparations as described in FCC[45]. In addition, Termamyl SC also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications[46].

The following specifications have been established for Termamyl SC:

α-amylase activity, KNU(S)/g	according to declaration
Heavy metals	not more than 30 ppm
Lead	not more than 5 ppm
Arsenic	not more than 3 ppm
Total viable count/g	not more than 5×10^4
Total coliforms/g	not more than 30
Enteropathogenic *E. coli*/25 g	negative by test
Salmonella/25 g	negative by test
Antibiotic activity	negative by test
Production organism	negative by test

6. APPLICATION

6.1 Mode of Action

Termamyl SC is a heat-stable α-amylase, which can operate at lower pH and lower calcium concentrations than conventional heat-stable α-amylases. The enzyme hydrolyzes 1,4-α-glucosidic linkages in amylose and amylopectin, forming soluble dextrins and oligosaccharides.

6.2 Application

In the starch industry, Termamyl SC is an α-amylase used for the liquefaction of starch in the production of syrups[28-31,47,48].

In the alcohol industry, Termamyl SC is an α-amylase used for thinning of starch in distilling mashes[28,49].

6.3 Use Levels

The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following Good Manufacturing Practices (GMP).

The recommended dosage for Termamyl SC is 0.45 kg per ton of starch. The optimum dosage depends on the desired effect and the specific circumstances in the processing plant.

6.4 Enzyme Residues in the Final Food

Termamyl SC is used as a processing aid and not added directly to food. Furthermore, the α-amylase enzyme protein is inactivated and removed during the conditions and steps used for industrial starch processing (high temperature (>100°C), low pH (<5), filtration, carbon treatment, and ion exchange)[28-31]. This has been confirmed by immunochemical (ELISA) analysis for the presence of residual enzyme protein in syrup samples taken at several stages in the normal starch hydrolysis process[50]. There was no detectable enzyme protein in the final syrup sample. The detection limit for the experimental assay is 710 pg/ml.

7. SAFETY EVALUATION

As stated most recently by FDA[51,52], issues relevant to a safety evaluation of an enzyme preparation are the safety of the enzyme source, enzyme component, manufacturing process, and a consideration of dietary exposure. Each of these is addressed below.

7.1 Safety of the Production Strain

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food[1]. If the organism is nontoxigenic and nonpathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, is safe to consume[6]. Pariza and Foster (1983) define a nontoxigenic 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 nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances". *B. licheniformis* is not a human pathogen and it is not toxigenic[15,53,54]. Carbohydrase

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and protease enzyme mixtures from *B. licheniformis* have been affirmed by the FDA as Generally Recognized as Safe[2] based partly on published literature establishing that *B. licheniformis* is widely recognized as a harmless contaminant of food and unpublished studies which corroborate that food uses of enzyme products from *B. licheniformis* are safe[15].

An evaluation of the genetically modified *B. licheniformis* production microorganism for Termamyl SC, according to the concepts initially outlined by Pariza and Foster (1983) and further developed by IFBC[6] in 1990, the EU SCF[7] in 1991, the OECD[8] in 1992, FAO/WHO[9] in 1996, and ILSI Europe Novel Food Task Force[10] in 1996, demonstrates the safety of this genetically modified production microorganism strain. The components of this evaluation: the identity of the host strain, a description of the plasmid used, the sources and functions of the introduced genetic material, an outline of the genetic construction of the production strain, and some characteristics of the production strain and the enzyme derived from it are given in Sections 2 and 3. Because the genetic modifications are well characterized and specific (see Sections 2 and 3), and the incorporated plasmid DNA is not known to encode or express any harmful or toxic substances, the Termamyl SC α-amylase enzyme preparation derived from the genetically modified *B. licheniformis* is considered safe.

The recipient host *B. licheniformis* strain, SJ1707, used in the construction of the Termamyl SC α-amylase production strain is a sporulation deficient and alkaline protease negative derivative of a natural isolate of *B. licheniformis*, ATCC 9789. As stated in Section 2, strain ATCC 9789 is the ancestor of *B. licheniformis* strains that have been used safely for industrial production of enzymes since 1972 including an enzyme preparation that was the subject of the GRAS affirmation petition[14] submitted by Novo Nordisk and affirmed by FDA in 1983[15].

B. licheniformis strain, SJ1707, has been used as a common host strain for several years in the construction of production strains for several other Novo Nordisk enzyme products including, SP844, SP703, SP501 and Termamyl LC. The SP844 production strain contains multiple copies of the wild-type *B. licheniformis* α-amylase gene, the SP703 and Termamyl LC production strains each contain a distinctly modified *B. licheniformis* α-amylase gene, and the SP501 production strain contains a wild-type *Thermoanaerobacter* sp. cyclodextrin glycosyl transferase gene. The SP844 production strain is used to make α-amylase for the starch and detergent industries. The SP703 production strain is used to make a modified α-amylase

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enzyme for the detergent industry. The SP501 production strain is used to make cyclodextrin glycosyl transferase for the starch industry. The Termamyl LC production strain is used to make a modified α-amylase enzyme for the starch industry.

For each of the above mentioned production strains, *B. licheniformis* strain SJ1707 was used as a common host strain for the construction of the final production strain by standard transformation procedures using well-known plasmid vectors and well-characterized DNA sequences. In each case, the recombinant plasmid DNA was integrated into the *B. licheniformis* host strain chromosome by homologous recombination. All of these production strains comply with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms[23] and meet the criteria for a safe production microorganism as described by Pariza and Foster (1983) and several expert groups[5-10]. Toxicological testing, confirming the safety of enzyme preparations derived from these *B. licheniformis* production strains, was done as indicated in the table below. An X indicates the study was done, a - indicates the study was not done.

Test substance	SP844	SP703	SP501	Termamyl LC
Batch number	PPY 5499	PPY 5067	PPA 4357	PPY 5977
Geno tox:				
Ames test	x	x	x	x
Chromosome aberration test	x	x	x	x
Mouse lymphoma test	-	-	x	-
In vivo tox:				
4-week oral in rats	x	x	-	x
13 week oral in rats	-	-	x	-
Comments	No treatment related effects	No treatment related effects	No treatment related effects	No treatment related effects
NOAEL/NOEL	1.32 g TOS/ kg bw /day	1.8 g TOS/ kg bw /day	2.6 g TOS/ kg bw/day	0.58 g TOS/ kg bw/day

The chromosome aberration test is an *in vitro* cytogenetic test using cultured human lymphocytes in the presence or absence of S-9 mix. The mouse lymphoma test is a gene mutation assay testing for the ability to induce mutations at the tk locus in mouse lymphoma cells in the presence or absence of S-9 mix.

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No toxicological effects were observed for any of the test substances produced by strains derived from *B. licheniformis* strain SJ1707. An independent expert panel has reviewed the toxicological information on SP501 and concluded that cyclodextrin glycosyl transferase enzyme preparation from the SP501 *B. licheniformis* production strain can be generally recognized as safe[55]. A summary of the toxicity data on Termamyl LC[56] has been submitted to the FDA Office of Premarket Approval as part of the GRAS notification documentation for Termamyl LC[57].

As stated above, the Termamyl SC production strain, LiH1464, was constructed using the same basic molecular and genetic techniques used for the other *B. licheniformis* strain SJ1707 derived strains. All of these production strains meet the criteria for a safe production microorganism as described by several expert groups[1,5-10]. The outcome of the appropriate safety evaluations outlined by these expert groups supports the determination that the Termamyl SC production strain LiH1464, containing the well-characterized introduced DNA sequences described in Section 2 and 3 that are not known to encode or express a harmful or toxic substance, would not present a pathogenic or toxigenic risk greater than that of its "parent" strain, SJ1707, or any of its derivatives described above and therefore can be regarded as a safe production microorganism for the modified *B. stearothermophilus* α-amylase enzyme.

7.2 Safety of the α-Amylase Enzyme

Enzyme proteins themselves do not generally raise safety concerns[1,51,58]. As shown in Section 3, Termamyl SC is an α-amylase, IUB EC 3.2.1.1, catalyzing the degradation of starch by hydrolysis of 1,4-α-glucosidic linkages in amylose and amylopectin.

7.2.1 α-Amylases

There is a long history of safe use of α-amylases from several microbial source organisms, including *B. licheniformis*[1]. In particular, bacterial α-amylases have been used in the starch industry since the 1950s[30] and, more recently, thermostable α-amylases from *B. licheniformis* and *B. stearothermophilus* have been used[28-31]. In 1973, GRAS petition 3G0026[14], which proposed the affirmation of the GRAS status of carbohydrase and protease preparations from *B. licheniformis*, was accepted for filing by the FDA[14]. Carbohydrases from *B. licheniformis* have been marketed in the US as GRAS since that time. In 1983, the FDA affirmed that mixed carbohydrase and protease enzyme product derived from *B. licheniformis* is GRAS when used to hydrolyze starches and

proteins in the production of certain foods[15]. In addition, α-amylase from *B. stearothermophilus* has been affirmed by FDA as GRAS[3] and FDA has published a notice of filing of a petition for affirmation of GRAS status for an α-amylase enzyme preparation derived from *B. licheniformis* expressing a *B. stearothermophilus* α-amylase[4].

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7.2.2 Substantial Equivalence

Several expert groups, as well as FDA and FDA scientists have discussed the concept of substantial equivalence relative to food safety assessment[5,8-13,59]. Essentially all these groups conclude that if a food ingredient is substantially equivalent to an existing food ingredient known to be safe, then no further safety considerations other than those for the existing ingredient are necessary.

In addition, FDA has applied this concept in the determination that several enzyme preparations are safe for use in food[12,60,61]. In particular, differences in glycosylation between enzyme proteins was considered. FDA has also stated that enzyme proteins demonstrated to be substantially equivalent to enzymes known to be safely consumed but having differences in specific properties due to changes in the enzyme amino acid sequence by natural selection, chemical modification, or site-directed mutagenesis would not raise safety concerns[11,13].

The Termamyl SC α-amylase enzyme from the genetically modified *B. licheniformis* is substantially equivalent to α-amylase from an unmodified *B. stearothermophilus.* Termamyl SC α-amylase is functionally equivalent and within the range of natural variation of *B. stearothermophilus* α-amylases (See section 3).

As noted by Kessler et al (1992), there is variation, due to genetic polymorphism, in DNA and amino acid sequence, protein structure, and optimal conditions of use among enzymes that have the same fundamental catalytic activity. α-Amylases from different strains of *B. stearothermophilus* exhibit slightly different properties[62] and have nearly, but not exactly, identical amino acid sequences (see Section 3 and Appendix B).

Termamyl SC has been modified to have increased stability at lower calcium concentrations and lower pH than naturally occurring *B. stearothermophilus* α-amylases (see Section 3). However, the properties and amino acid sequence of Termamyl SC α-amylase are within the range of naturally occurring biological diversity of *B. stearothermophilus* α-amylases (see Section 3). Termamyl SC has the same molecular weight, >96% amino acid identity, and

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significantly similar structure to other *B. stearothermophilus* α-amylases. In addition, Termamyl SC has the same functional activity as other *B. stearothermophilus* α-amylases (see Section 3 and Appendices D and E).

Novo Nordisk and other enzyme manufacturers have produced and marketed significant amounts of modified enzymes for non-food technical applications such as use in laundry detergents for several years, the first one developed in 1991[63,64,Appendix H]. These modified enzymes are substantially equivalent to traditional microbially derived proteases, amylases and lipases. Novo Nordisk has communicated with the US Environmental Protection Agency regarding the manufacture and use of these enzymes. These and other manufacturer's modified products have been safely manufactured and used.

The Termamyl SC α-amylase is substantially equivalent to other *B. stearothermophilus* α-amylases and is safe for use in food.

7.3 Safety of the Manufacturing Process

Termamyl SC meets the general and additional requirements for enzyme preparations as outlined in the monograph on Enzyme Preparations in the Food Chemicals Codex[45]. As described in Section 4, the α-amylase preparation is produced in accordance with current good manufacturing practices, using ingredients that are acceptable for general use in foods, and under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for the production of microbial enzymes[65-67].

7.4 Potential Dietary Exposure

7.4.1 Exposure from Use in Industrial Starch Processing

As stated in Section 6.4, Novo Nordisk has experimentally verified that the Termamyl LC α-amylase enzyme protein is inactivated and removed during the conditions and steps used for industrial starch processing. Therefore, the potential human exposure to the enzyme from the consumption of food is negligible.

7.4.2 Exposure from Use in the Production of Alcohol

The enzyme is used in the early stages of alcohol production. It is added to the starch, which is subjected to further processing steps, including hydrolysis to fermentable sugars. The sugars are fermented

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to produce alcohol and the alcohol is distilled. During these steps the enzyme is inactivated. Inactivated enzyme is not transferred with the distillate, and thus it is not possible for enzyme or enzyme residues to be present in the final alcohol.

7.4.3 Enzyme Exposure Safety Assessment

In addition, any dietary exposure to an enzyme preparation used in food processing that is derived from a controlled fermentation of a nonpathogenic, nontoxigenic microorganism that does not produce antibiotics, and that is manufactured using substances that are acceptable for general use in foods, would not ordinarily present a basis for safety concern[13]. Termamyl SC α-amylase meets these criteria and is safe for use in food.

7.5 Results and Conclusion

On the basis of the evaluation contained in Sections 7.1 - 7.4, a review of the published literature, the history of use of *B. licheniformis* and *B. licheniformis* and *B. stearothermophilus* α-amylases, and the limited and well defined nature of the genetic modifications, the Termamyl SC α-amylase enzyme preparation produced by *B. licheniformis* expressing a gene encoding a modified α-amylase from *B. stearothermophilus* can be safely manufactured and used as a processing aid in the starch and alcohol industry.

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8. LIST OF APPENDICES

A. pLiH1346 plasmid map

B. Amino Acid Sequence Alignment and Comparison of *B. stearothermophilus* α-Amylases

C. Amino Acid Identity of *B. stearothermophilus* α-Amylases

D. Experiment Report- "Characterization of Termamyl SC action pattern"; Luna no. 1999-02399-01

E. Experiment Report- "Comparison of Starch liquefied with Termamyl Type S and Termamyl type SC", Luna no.:1999-02397-01

F. Manufacturing Process Flow Diagrams
Figure 1 Fermentation
Figure 2 Recovery

G. Novo Nordisk Product Sheet for Termamyl SC

H. Novo Nordisk Detergent Industry Product Range Sheet

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Appendix B

Appendices A and B: Redacted, Confidential Business Information
Pages 000034, 000036, and 000037

APPENDIX C

Amino Acid Identity of *B. stearothermophilus* α-Amylases

	Termamyl SC	A5451	A24549	A24436	AAB86961	AAA22227
Termamyl SC	100	99.4	98.4	97.5	99.0	96.5
A5451		100	99.0	98.1	99.6	97.1
A24549			100	98.3	99.4	97.3
A24436				100	98.4	98.4
AAB86961					100	97.5
AAA22227						100

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Application Technology 02-03-1999 MW
Starch degrading Enzymes
Luna no.: 1999-02399-01

To: SvP, BEN, KRyN, TCR, MpI
From: MPI/MW

Characterisation of Termamyl SC action pattern

Background

Thermostable α-amylases have been used for many years in the starch industry. They are used in the first step, liquefaction, in the processing of starch to for example glucose or fructose syrups. During liquefaction a starch slurry (typical 35 % DS[1]) is heated to 105 °C in a jet-cooker. To reduce the viscosity of the cooked starch, a thermostable α-amylase is added to the starch at the beginning of the liquefaction process. The holding time at 105 °C is 5 minutes, after which the starch is flash-cooled to about 95 °C. The enzyme, which is still active, continues the hydrolysis for up to two hours: After this treatment the starch will have a DE[2] of 10 - 15

The liquefaction process is completed before a significant starch hydrolysis has taken place. If the reaction is allowed to proceed, differences in action pattern, between different α-amylases, can be detected.
A thermostable α-amylase from *B. Stearothermophilus* will for example produce a big amount of maltohexaose.

To verify that Termamyl type SC has the same action pattern as the native enzyme, Termamyl Type S, a starch hydrolysis experiment was carried out and the carbohydrate spectrum analysed by HPLC.

Work carried out
5 g waxy corn starch (Cerestar 04201) was suspended in 100 ml 0.01 M acetate buffer containing 40 ppm $CaCl_2$ and boiled for 2 minutes under constant stirring. After cooling to about 70 °C 2 x 20 g of the slurry was weighed out in 50 ml blue cap flasks, magnetic stirrers were added to the flasks, which then was placed in a water bath at 60 °C.
When the starch slurry was equilibrated the enzyme was added.

The following enzyme were tested

Termamyl Type S	Batch AEP 30030	115 KNU (SM 9)[3] /g
Termamyl Type SC	Batch 98LSCo024	300 KNU (SM9)/g

[1] DS = Dry Substance
[2] DE (dextrose equivalent) % reducing sugars expressed as dextrose on dry solid basis
[3] Kilo Novo Units analysed according to Novo Nordisk standard method EAL-SM-0009

Trial conditions:

Substrate concentration	0.5 % w/w
Enzyme dosage	150 NU/g DS
pH	6.0
Calcium level	40 ppm.

Sampling

Samples were taken after 1,2, 4, 6, 24 and 48 hours.
2 ml sample was diluted in 2 ml Milli Q water containing 2 drops of 1 M HCl to inactivate the enzyme. The test tubes were mixed and boiled for 15 minutes.
After cooling the sample were filtered through a 0.2 µl filter and analysed by HPLC

Results

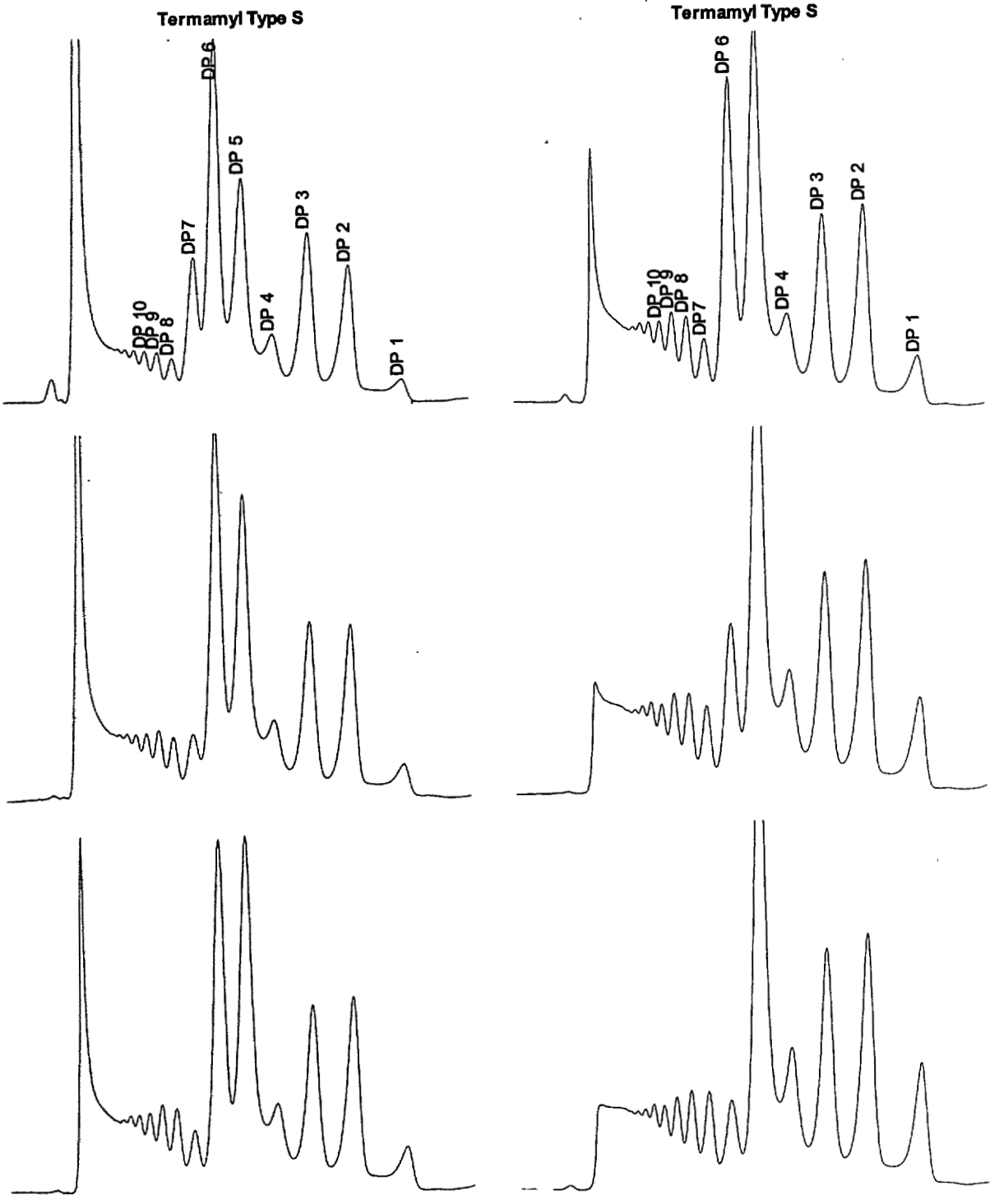
se attached HPLC chromatogram

Conclusion

The action pattern of Termamyl SC is identical to the action pattern of Termamyl type S.

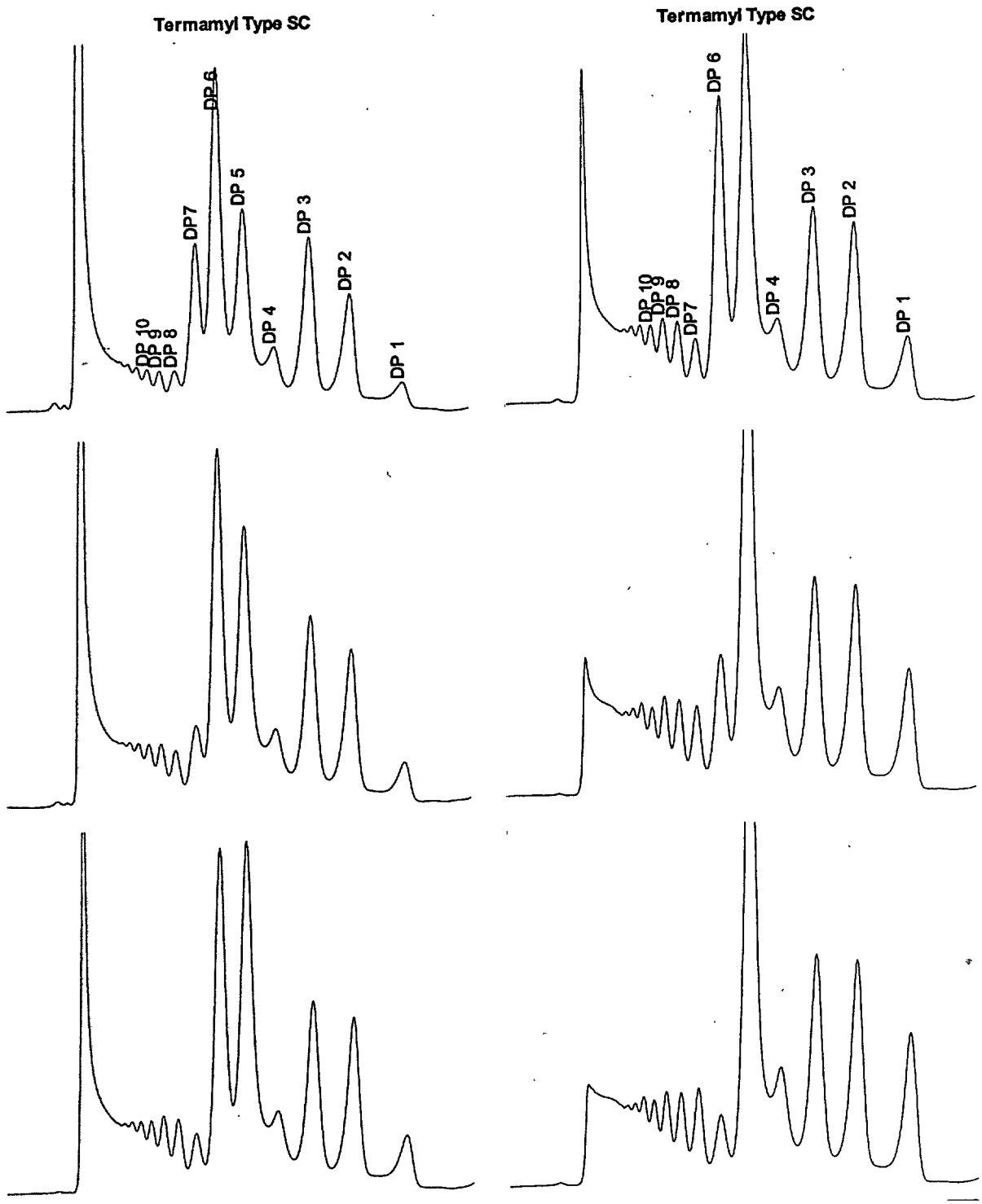
References

Study Plan no.	99MW005
Study memo	99MW005
Laboratory journal	08834 page 39
Standard procedure	ABF-5004.01
Standard method	ABF-SM-5122.02 Determination of sugars (DP_1 - DP_{10+}) in hydrolysed starch syrups by IMP HPLC.



1 hour
2 hours
4 hours

6 hours
24 hours
48 hours



1 hour
2 hours
4 hours

6 hours
24 hours
48 hours

Appendix E

Application Technology 1 1/3-1999/MW
Starch Degrading Enzymes
Luna no.: 1999-02397-01

To: SvP, BEN, KRyN, TCR, MPI
From: MW

Comparison between Starch liquefied with Termamyl Type S and Termamyl type SC

Purpose

In order to verify that starch liquefied with Termamyl type SC is essentially the same as starch liquefied with Termamyl type S, liquefaction trials, followed by saccharification were carried out.

Background

The conversion of starch to glucose consists of two hydrolytic stages : liquefaction and saccharification. During liquefaction, a starch slurry containing 30 - 40 % dry solids, is pumped through a "jet cooker" where the temperature is raised to typical 105 °C by direct steam injection. To reduce the viscosity of the cooked starch, a thermostable α-amylase is added to the starch at the beginning of the liquefaction process. The holding time at 105 °C is 5 minutes, after which the starch is flash-cooled to about 95 °C. The enzyme, which is still active, continues the hydrolysis for up to two hours: After this treatment the starch will have a DE[1] of 10 - 15.

After liquefaction the partially hydrolysed starch is saccharified to D-glucose, by the action of glucoamylase and pullulanase. The saccharification is carried out at 60 °C pH 4.3 for a period of 48-96 hours. The yield of D-glucose is dependent on the liquefaction process.
There might be losses due to by-product formation or to incomplete hydrolysis resulting from the choise of liquefying enzyme.

Work carried out

Liquefaction

Corn starch slurries were prepared by suspending 11.8 kg Cerestar C*pharm GL 03406 (89 % DS) in deionised water up to 30 kg. The pH was adjusted to 5.8 at ambient temperature, after the addition of 3.85 g $CaCl_2$ 2 H_2O.

[1] DE (dextrose equivalent) % reducing sugars expressed as dextrose on dry solid basis

Samples for carbohydrate composition

Samples were taken at 24, 48 and 72 hours. The samples were heated in a boiling water bath for 15 minutes to inactivate the enzyme. After cooling the samples were diluted 1:2 in milli-Q water and treated with mixed bed ion exchange resin (Bio Rad AG 501/X8 (D) for 30 minutes to remove ash and soluble N, and finally filtered through a 0.2 µm filter, before being analysed by HPLC.

Samples for measurement of immunochemical residual activity

During the experiment and after saccharification samples were taken in order to check for residual α-amylase activity. The samples were frozen immediately after being taken .
After saccharification, the saccharified starch was heated in a boiling water bath for 15 minutes, and filtered through a Wathman GFB filter. 350 ml of this solution were treated with activated charcoal (3 teaspoons), for 30 minutes, at 60 °C. The solution was then filtered through a 0.2 µm filter followed by 30 minutes ion exchange with mixed bed ion exchange resin (Bio Rad AG 501/X8 (D)) for 30 minutes, and finally filtered one more time through a 0.2 µm filter.

The samples are marked
S1: After liquefaction before inactivation
S2: After liquefaction after inactivation
S3: After saccharification
S4: After first filtration (Wathman GFB)
S5: After carbon treatment and filtration (0.22 µm filter)
S6: After ion exchange and filtration (0.22 µm filter)

Results carbohydrate composition

Liquefaction Termamyl type S (*B. stearothermophilus*)

Hours	% DP_1[5]	% DP_2	% DP_3	% DP_{4+}
24	94.7	2.2	1.0	2.2
48	96.5	2.2	0.6	0.7
72	96.4	2.7	0.4	0.5

Liquefaction Termamyl type SC (*B. stearothermophilus*)

Hours	% DP_1	% DP_2	% DP_3	% DP_{4+}
24	95.0	2.1	0.9	2.0
48	96.5	2.2	0.5	0.7
72	96.4	2.7	0.4	0.5

Results of measurement of immunochemical residual activity
see document no. 1999-0291-01

[5] Degree of polymersation, DP_1 monosaccharides, DP_2 disaccharides

The following enzymes were used:

Termamyl 120 L type S	Batch: AEP 30030	115KNU[2] (SM 9)/g
Termamyl type SC	Batch: 98LSCo024	300 KNU (SM 9)/g

The following amount of α-amylases were added

Termamyl Type S	50 KNU (SM 9) / kg DS
Termamyl type SC	50 KNU(SM 9) / kg DS

After addition of calcium and pH adjustment, the enzyme was added. The conductivity of the starch slurries were adjusted to 500 µS/cm and finally, if necessary, pH was adjusted to 5.8. again.

Substrate concentration	35 % w/w (initial) 31 % w/w (final)
Temperature	105 °C 5 minutes (primary liquefaction) 95 °C 120 minutes (secondary liquefaction)
pH (initial)	5.8
Calcium level	35 ppm

After liquefaction, pH was lowered to ≈ 3,5 -4,0 for 10 minutes to inactivate the enzyme.

Saccharification

485 g of substrate (equivalent to 150 g DS liquefied starch) was transferred to a 500 ml blue cap flask and placed in a water bath at 60 °C. Magnetic stirrers were added to the flask.
After the liquefied starch was equilibrated to 60 °C pH was adjusted to 4.3 and the saccharification enzymes added. pH was adjusted again 15 minutes after the addition of enzyme and again after 24 hours.

Enzyme used:	Dextrozyme E	183 AGU [3]/g 370 NPUN[4]/g
dosage		0.16 AGU, 0.32 NPUN / g DS
Substrate concentration		31 % w/w (initial) 33.5 % w/w (Final)
pH initial		4.3
Temperature		60 °C

[2] Kilo Novo Units analysed according to Novo Nordisk standard method EAL-SM-0009
[3] Amyloglucosidase unit according to Novo Nordisk standard method EAL-SM-0022.01
[4] New pullulanase Unit Novo according to Novo Nordisk standard method KAL-SM-0420.01

Conclusion

There is no detectable difference in glucose yield or carbohydrate composition after saccharification between starch liquefied with Termamyl Type S or Termamyl Type SC

References

Study plan no:	99MW002
Study memo no:	99MW002
Laboratory journal no	6922 page 081 -090 08834 page 14-23
Standard Procedure:	ABF-5046.01 forflydning af stivelse i pilot plant (Liquefaction in pilot plant)
Standard procedure:	ABF-5004.01 Forsukringsforsøg (saccharification)
Standard method:	ABF-SM-5160.01 Bestemmelse af dextrose equivalent (Determination of DE)
Standard method:	ABF-SM-5121.02 Determination of Sugars (DP_1 - DP_{4+}) in hydrolysed Starch Syrups by IMP HPLC.

Appendix F

Termamyl® SC

Fermentation

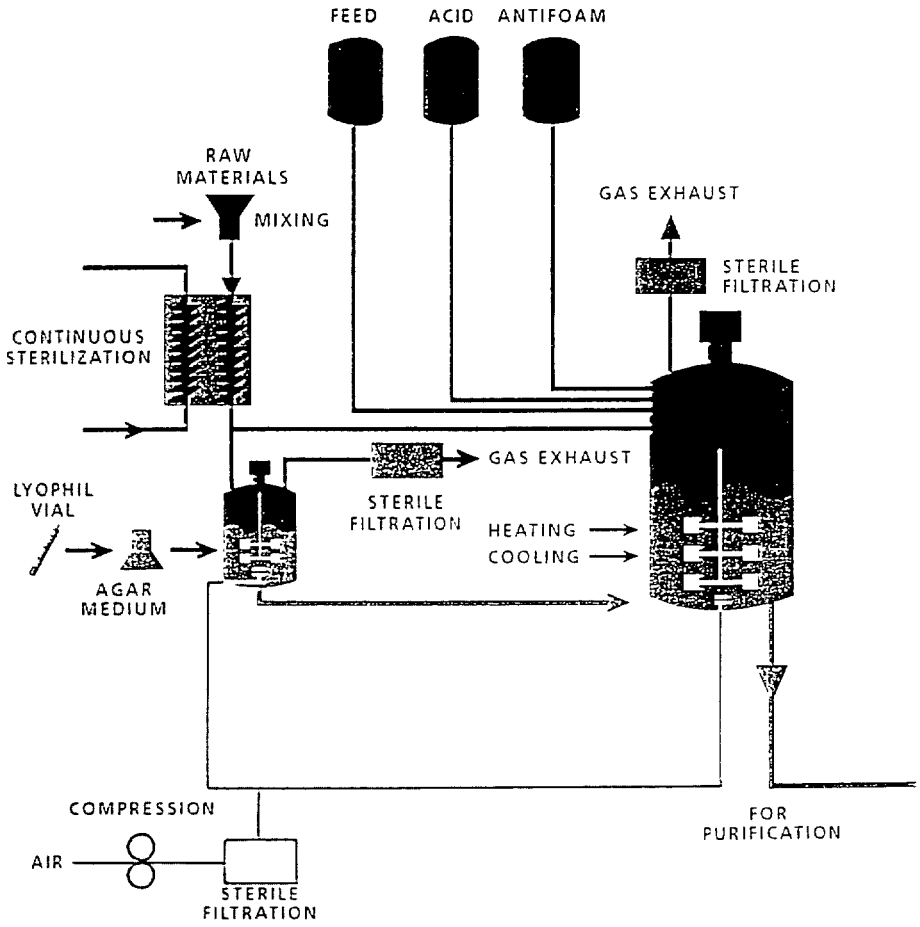


FIGURE 1

Termamyl® SC

Recovery

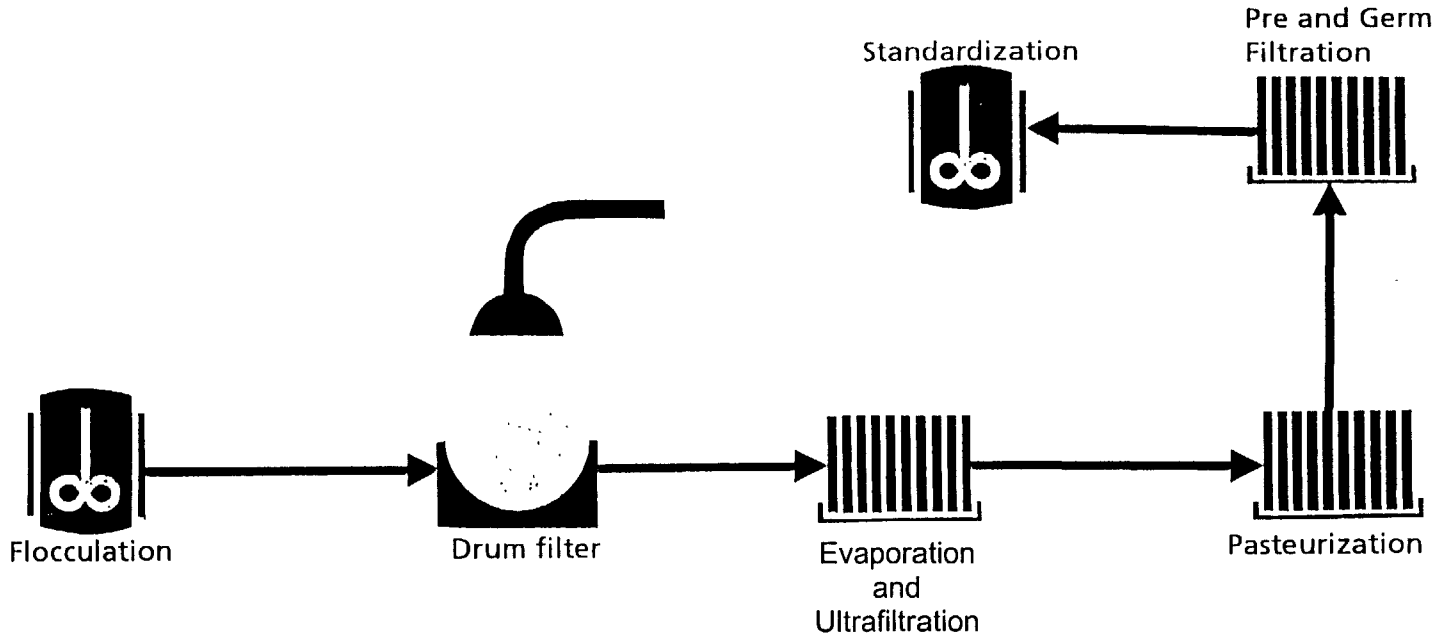


Figure 2

Appendix G

Termamyl® SC

Description

Termamyl SC is a liquid enzyme preparation containing an outstandingly heat-stable alpha-amylase expressed in and produced by a genetically modified strain of a *Bacillus* microorganism.

The systematic name for the enzyme is 1,4-α-D-glucan glucano-hydrolase (EC 3.2.1.1).

Termamyl SC is a remarkable new liquefaction enzyme which can operate at lower pH and calcium levels than conventional thermostable alpha-amylases. This brings several advantages to its applications which can all be translated into reduced operating costs.

Specification

Appearance

Termamyl SC is a brown liquid with a density of 1.20-1.25 g/ml.

Activity

Termamyl SC is available with a standard strength of 120 KNU/g.
A detailed description of Novo Nordisk's method of analysis is available on request.

Packaging

Termamyl SC is available in 30 kg jerry cans, 250 kg steel drums, 1200 kg Schütz containers and in bulk by the truck-load.

Application

The enzyme is an endo-amylase which hydrolyzes 1,4-alpha-glucosidic linkages in amylose and amylopectin. This results in a rapid reduction of the viscosity of gelatinized starch. The breakdown products are dextrins of different chain lengths, and oligosaccharides.

Termamyl SC is used for thinning starch-containing mashed or starch slurries in the production of neutral spirits. Due to its broad pH tolerance and low calcium requirement, pH adjustment and calcium addition can often be omitted. This simplifies the process and reduces calcium oxalate scaling.

Detailed recommendations concerning the application of Termamyl SC are given in an Application Sheet.

Safety

Enzymes are proteins and inhalation of dust or aerosols may induce sensitization and may cause allergic reactions in sensitized individuals.
Some enzymes may irritate the skin, eyes and mucous membranes upon prolonged contact.
The product may create easily inhaled aerosols if splashed or vigorously stirred. Spilled product may dry out and create dust.
Spilled material should be flushed away with water (avoid splashing).
A Material Safety Data Sheet and separate material describing how to handle the product safely are available on request.

Storage

Enzymes gradually lose activity over time depending on storage temperature. Cool conditions are recommended.
When stored in closed containers at 5°C, Termamyl SC will maintain its declared activity for 6 months.
Extended storage and/or adverse conditions, including higher temperature, may lead to higher dosage requirements.

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Laws, regulations and third party rights may prevent customers from importing, processing, applying and/or reselling certain products in a given manner. It is the responsibility of the customers that their specific use of products from Novo Nordisk does not infringe relevant laws and regulations and, furthermore, does not infringe patents or other third party rights.

The contents of this document are subject to change without further notice.

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Appendix H

Detergent Industry

Proteases

Alcalase®
A bacterial protease effective under neutral and mildly alkaline conditions (pH 7-10). Suitable for soaking preparations and liquid as well as powder detergents. Alcalase is also characterized by being non aggressive towards wool.

Savinase®
A bacterial protease which is effective under alkaline conditions (pH 8-11). Savinase is the most commonly used protease and works well under most washing conditions.

Everlase™
A protein-engineered variant of Savinase characterized by having an excellent storage stability in bleach-containing detergents.

Esperase®
A bacterial protease which is effective under strongly alkaline conditions (up to pH 12 approximately) even at very high temperatures.

Lipases

Lipolase®
A fungal lipase. Lipolase is effective under alkaline conditions (up to pH 12 approximately) and at a broad temperature range.

Lipolase Ultra
A protein-engineered variant of Lipolase. In general, the washing performance of Lipolase Ultra is superior to that of Lipolase at high ionic strength, low temperatures and high pH, and various combinations of these conditions.

LipoPrime™
A protein-engineered variant of Lipolase. LipoPrime shows superior fat-removing efficacy in comparison to Lipolase and Lipolase Ultra in most detergent formulations.

Amylases

Termamyl®
A bacterial amylase which is able to work at relatively high (alkaline) pH values (up to pH 11) and at high temperatures (up to 100°C)

BAN
A traditional bacterial amylase which is effective at neutral and mildly alkaline conditions and at low and moderate wash temperatures. (Only available as a co-granulate with Savinase.)

Duramyl®
A protein-engineered variant of Termamyl characterized by having an excellent storage stability in bleach-containing detergents as well as superior performance at medium temperatures compared to Termamyl.

Cellulases

Celluzyme®
A fungal enzyme product containing a cellulase complex active in the neutral to moderately alkaline region Celluzyme is well-suited for washing temperatures from 15°C to 65°C and it works on garments made from cellulosic fibres, as for example cotton and cotton blends.

Carezyme®
A monocomponent cellulase showing remarkable colour brightening benefits that works on cotton and cotton-containing blends. Carezyme is effective under neutral to moderately alkaline conditions. Besides it is well-suited for washing temperatures ranging from 15°C to 65°C

Product Sheets available on request.

Novo Nordisk

SUBMISSION END

AM

6 July, 1999

Mary Ditto, Ph.D.
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RE: GRAS Notice No. GRN 000024

Dear Dr. Ditto,

As per our recent telephone conversation regarding Novo Nordisk's GRAS Notice No. GRN 000024 for an α-amylase enzyme preparation produced by submerged fermentation of *Bacillus licheniformis* carrying a modified gene for α-amylase from *Bacillus stearothermophilus*, please find two pages (numbered 6 and 20) containing corrections to the originally submitted binder containing summary information supporting the GRAS determination.

The corrections on page 6 include, the change from "was" to "were" in the first sentence of section 2.4; substitution of the more appropriate phrase "during cellular growth and reproduction," for "mitotically" in the last sentence of section 2.4; and the change to "Termamyl SC" instead of "Termamyl LC" in the first sentence of section 2.6. The correction on page 20 is the change to "Termamyl SC" instead of "Termamyl LC" in the first sentence of section 7.4.1.

Please contact me by direct telephone at 919 494-3152 or direct fax at 919 494-3420 if you have any questions or require additional information.

Sincerely,

Scott H. Shore, Ph.D.
Senior Regulatory Specialist

1999 JUL -8 P 2:41

Enclosures (2 pages)

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2.4 Stability of the Transformed Plasmid Genetic Sequences

The presence and configuration of the introduced DNA sequences were determined by Southern hybridization to assess the stability and potential for transfer of genetic material as a component of the safety evaluation of the production microorganism[5-10]. The transforming plasmid DNA sequences are stably integrated into the *B. licheniformis* chromosome and do not contain any sequences known to promote gene transfer. The introduced genetic material is poorly mobilizable for genetic transfer to other organisms and is stable during cellular growth and reproduction.

2.5 Absence of Transformable rDNA in the Enzyme Preparation

The absence of transformable recombinant DNA sequences used in the production strain construction is a component of the safety evaluation of the production microorganism[6,7]. A sample of the Termamyl SC enzyme preparation was analyzed for the presence of transformable DNA sequences[25]. The analysis was based on selection of kanamycin resistant transformants. No transforming DNA capability was detected in the sample of the Termamyl SC enzyme preparation analyzed. Therefore, there is no potential for transfer of recombinant DNA sequences to other organisms which ingest foods processed with the α-amylase enzyme preparation.

2.6 Absence of Production Organism in the Product

The absence of the production organism is an established specification for the commercial product, Termamyl SC. The production organism does not end up in food and therefore the first step in the safety assessment as described by IFBC[6] is satisfactorily addressed.

2.7 Antibiotic Resistance Gene

Plasmid pLiH 1346 contains a kanamycin resistance gene, encoding an aminoglycoside 3'-phosphotransferase II protein, APH(3') II. FDA has evaluated the safety of this gene and gene product for use in the development of genetically engineered tomato, oilseed rape, and cotton[26]. The FDA's evaluation of issues and the agency's conclusions are also applicable for assessment of the kanamycin resistance gene present in the Termamyl SC production strain. As discussed by FDA in its decision[26] and further elaborated in the draft guidance and report on the use of antibiotic resistance marker genes in transgenic plants[27], the evaluation of the safety of the use of an antibiotic resistance marker gene should include an assessment of

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significantly similar structure to other *B. stearothermophilus* α-amylases. In addition, Termamyl SC has the same functional activity as other *B. stearothermophilus* α-amylases (see Section 3 and Appendices D and E).

Novo Nordisk and other enzyme manufacturers have produced and marketed significant amounts of modified enzymes for non-food technical applications such as use in laundry detergents for several years, the first one developed in 1991[63,64,Appendix H]. These modified enzymes are substantially equivalent to traditional microbially derived proteases, amylases and lipases. Novo Nordisk has communicated with the US Environmental Protection Agency regarding the manufacture and use of these enzymes. These and other manufacturer's modified products have been safely manufactured and used.

The Termamyl SC α-amylase is substantially equivalent to other *B. stearothermophilus* α-amylases and is safe for use in food.

7.3 Safety of the Manufacturing Process

Termamyl SC meets the general and additional requirements for enzyme preparations as outlined in the monograph on Enzyme Preparations in the Food Chemicals Codex[45]. As described in Section 4, the α-amylase preparation is produced in accordance with current good manufacturing practices, using ingredients that are acceptable for general use in foods, and under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for the production of microbial enzymes[65-67].

7.4 Potential Dietary Exposure

7.4.1 Exposure from Use in Industrial Starch Processing

As stated in Section 6.4, Novo Nordisk has experimentally verified that the Termamyl SC α-amylase enzyme protein is inactivated and removed during the conditions and steps used for industrial starch processing. Therefore, the potential human exposure to the enzyme from the consumption of food is negligible.

7.4.2 Exposure from Use in the Production of Alcohol

The enzyme is used in the early stages of alcohol production. It is added to the starch, which is subjected to further processing steps, including hydrolysis to fermentable sugars. The sugars are fermented

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