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Original Submission

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29 June, 2001

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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

JUL 03 2001

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 Novozymes' α-amylase enzyme preparation (trade name Novozym 28035), produced by *Bacillus licheniformis* carrying the gene encoding a modified α-amylase from *Bacillus licheniformis*. The α-amylase enzyme preparation is to be used as a processing aid in the starch industry (for the liquefaction of starch in the production of syrups) and in the alcohol industry (for the thinning of starch in distilling mashes).

Please contact me by direct telephone at 919 494-3150 or direct fax at 919 494-3420 if you have any questions or require additional information.

Sincerely,

John Carroll
Director, Regulatory Affairs

Enclosures (3 binders)

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29 June, 2001

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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) Novozymes North America, Inc. hereby claims that an α-amylase enzyme preparation (trade name Novozym 28035), produced by *Bacillus licheniformis* carrying the gene encoding a modified α-amylase from *Bacillus licheniformis* is Generally Recognized as Safe; therefore, it is exempt from statutory premarket approval requirements.

The α-amylase enzyme preparation is to be used as a processing aid in the starch industry (for the liquefaction of starch in the production of syrups) and in the alcohol industry (for the thinning of starch in distilling mashes).

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

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

Novozymes 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 licheniformis*.

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

The above described α-amylase preparation is to be used as a processing aid in the starch industry (for the liquefaction of starch in the production of syrups) and in the alcohol industry (for the thinning of starch in distilling mashes). 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.

John Carroll — Date
Director, Regulatory Affairs

Luna: 2001-10066-01

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GRAS NOTIFICATION

Novozym 28035

an α-amylase preparation

produced by *Bacillus licheniformis* expressing

a gene encoding a modified

α-amylase from *Bacillus licheniformis*

Scott Shore, Enzyme Regulatory Affairs, Novozymes North America, USA
Kim Rygaard Nielsen, Enzyme Regulatory Affairs, Novozymes A/S, Denmark

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1. GENERAL INTRODUCTION

Novozym 28035 is the Novozymes A/S trade name used for an α-amylase enzyme preparation produced by submerged fermentation of a genetically modified strain of *Bacillus licheniformis* which contains a modified *B. licheniformis* α-amylase gene.

The enzyme preparation is to be used as a processing aid in the starch industry (for the liquefaction of starch in the production of syrups) and in the alcohol industry (for the thinning of starch in distilling mashes).

The enzyme is a heat-stable α-amylase (EC 3.2.1.1), 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 information contained in this document presents the basis for Novozymes' determination of the general recognition of safety of an α-amylase enzyme preparation produced by a genetically modified strain of *B. licheniformis* expressing a modified *B. licheniformis* α-amylase gene. Sections 2-7 provide information on the enzyme, the production microorganism, the manufacturing process, product specifications, the intended use, and an overall safety evaluation.

The overall safety evaluation (Section 7) includes a review of published and unpublished information supporting the safety of the production strain, the enzyme, and the manufacturing process as well as an evaluation of potential dietary exposure. A summary of the toxicology studies done to confirm the safety of the product is also included. This overall safety evaluation concludes that the α-amylase enzyme preparation is safe for its intended use.

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[1,2]. The production organism for Novozym 28035, a genetically modified strain of *B. licheniformis* expressing a modified *B. licheniformis* α-amylase gene, is discussed in sections 2 and 7. 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[2-8]. The methods used to develop the genetically modified production organism and the specific genetic modifications introduced into the production organism are described in Section 2. Genetically modified microbial production strains derived from an established safe strain lineage and can be regarded as

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safe[2,4]. Information is presented in Sections 2 and 7 demonstrating that the genetically modified production organism for Novozym 28035 is part of a safe strain lineage established by Novozymes. The safety of this production microorganism is considered in Section 7 and it is concluded to be safe.

Section 3 includes a description of the α-amylase activity and a summary of data showing this modified α-amylase to be substantially equivalent[6-11] to naturally occurring and other genetically modified *B. licheniformis* α-amylases previously determined to be Generally Recognized As Safe (GRAS).

The manufacturing process, including the raw materials, processing aids, and other substances used, is described in Section 4. The composition and specifications of the commercial product, Novozym 28035, are given in Section 5.

Section 6 describes the intended use for Novozym 28035, its technical function, recommended 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.

This compilation of information addresses the safety of the enzyme source, the enzyme component, the manufacturing process, and a consideration of dietary exposure, all the issues relevant to a safety evaluation of an enzyme preparation, as stated most recently by FDA[12-13]. Based on critical review and evaluation of this published and unpublished information, Novozymes concludes, through scientific procedures, that the described *B. licheniformis* α-amylase preparation, meeting appropriate food grade specifications and produced in accordance with current good manufacturing practices, is GRAS for the intended conditions of use.

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2. PRODUCTION MICROORGANISM

2.1 Production Strain

The microbial production strain for Novozym 28035, designated MOL2803, 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 marketed and sold by Novozymes since 1972, including an enzyme preparation that was the subject of the GRAS affirmation petition[14] submitted by Novozymes (formerly Novo Nordisk) and affirmed by FDA in 1983[15]. The classification of the ATCC strain and the production microorganism is based on generally accepted taxonomic characteristics[16].

The construction of *B. licheniformis* strain MOL2803[17] was done using common bacterial transformation and conjugation procedures with well-known plasmid vectors[18-20] and well-characterized, strictly defined *B. licheniformis* chromosomal DNA sequences[21-22]. Most significantly, no heterologous DNA sequences were incorporated in the production strain. The only incorporated DNA sequences were *B. licheniformis* chromosomal DNA sequences and the modified *B. licheniformis* α-amylase gene. The *B. licheniformis* production strain MOL2803 has three copies of the modified *B. licheniformis* α-amylase gene integrated at three distinct sites on the *B. licheniformis* chromosome. The modified α-amylase DNA sequences were integrated into the *B. licheniformis* host strain chromosome at these sites by homologous recombination. The identity and location of the three inserted copies of the modified *B. licheniformis* α-amylase gene were verified by polymerase chain reaction (PCR) amplification followed by DNA sequencing of the amplified fragments. The *B. licheniformis* α-amylase gene (amyL) was modified by well-known techniques designed to introduce specific desired changes in the coding sequence[23-25]. The identity and location of these changes were verified by DNA sequencing in both orientations several times. The genetic construction of *B. licheniformis* strain MOL2803 was evaluated at every step to assess the incorporation of the desired functional genetic information and to ensure that no unintended sequences were incorporated in the production strain.

The genetically modified *B. licheniformis* production strain MOL2083 is nonpathogenic nontoxigenic and meets the criteria for a safe production microorganism as described by Pariza and Foster (1983), Pariza and Johnson (2001), and several expert groups[3-8]. These criteria include the identification and characterization of the host strain, plasmid vectors, genetic modification techniques, and inserted genetic sequences, all described below. In addition, the *B. licheniformis* production strain MOL2083 complies with the OECD

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(Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms[26].

2.2 Initial Host Strain

The *B. licheniformis* initial host strain, designated SJ1707, used in the construction of the Novozym 28035 α-amylase production strain is a sporulation deficient and alkaline protease negative derivative of a natural isolate of *B. licheniformis*, ATCC 9789.

2.3 Introduced DNA Sequences

The only DNA sequences introduced were *B. licheniformis* chromosomal DNA sequences and the modified *B. licheniformis* α-amylase gene. The modified *B. licheniformis* α-amylase gene contains a segment of *B. licheniformis* chromosomal DNA found immediately 5' upstream of the *amyL* gene promoter denoted UPS P*amyL*, a strong *amyL* promoter variant denoted P_{4199}, the *amyL* signal peptide sequence, the coding sequence for the modified α-amylase denoted Novozym 28035, and the 3' downstream region of the *amyL* gene. The modified *B. licheniformis* α-amylase gene coding sequence and the amino acid sequence of the mature enzyme protein is described more fully in Section 3.2.

2.4 Construction of the Production Strain MOL2083

Bacillus licheniformis strain SJ1707 was used as the initial host strain. Three copies of the modified *B. licheniformis* α-amylase gene were integrated in three separate steps at three distinct sites on the *B. licheniformis* chromosome. Integration occurred by double crossover homologous recombination at *amyL*[22], *xyl* (xylose isomerase locus)[27], and *gnt* (gluconate permease locus)[28] after using bacterial conjugation to transfer a plasmid containing the modified Novozym 28035 α-amylase gene flanked by either *amyL*, *xyl*, or *gnt B. licheniformis* chromosomal DNA sequences. In a separate step, the C-component protease gene was deleted by double crossover homologous recombination with a DNA fragment containing sequences upstream of the C-component protease fused to sequences downstream of the C component protease, thus lacking the C-component protease coding region. The resulting sporulation deficient, alkaline protease negative, C-component protease negative strain containing three copies of the modified α-amylase gene was designated MOL2083.

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2.5 Stability of the Introduced DNA Sequences

As indicated in Section 2.1, the identity and location of the three inserted copies of the modified *B. licheniformis* α-amylase gene were verified by PCR amplification followed by DNA sequencing of the amplified fragments[17]. In addition, the presence and configuration of the three inserted copies of the modified *B. licheniformis* α-amylase gene were determined by Southern hybridization[17]. The three inserted copies of the modified *B. licheniformis* α-amylase gene are stably integrated into the *B. licheniformis* chromosome. The three inserted copies of the modified *B. licheniformis* α-amylase gene do not contain any sequences known to promote gene transfer. The introduced DNA sequences are integrated in the host *B. licheniformis* chromosome and are therefore poorly mobilizable for genetic transfer to other organisms and are stable during cellular growth and reproduction.

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3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1 Enzyme Identity

Key characteristics of Novozym 28035 enzyme are given below. It is an α-amylase as defined by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology[29].

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

The Novozym 28035 modified α-amylase has been developed to improve enzyme performance by increasing stability at low pH, low calcium concentration, and high temperature. The starting point for the Novozym 28035 modified α-amylase was the modified *B. licheniformis* α-amylase gene used in the production of Novozymes' product Termamyl LC (subject of GRAS Notification GRN 000022)[30]. The Termamyl LC α-amylase gene was constructed by splicing the NH_2-terminal region of the *B. amyloliquefaciens* α-amylase sequence into the wild-type *B. licheniformis* α-amylase gene and changing the DNA coding sequence for five other specific amino acid residues. The complete amino acid sequence for the Termamyl LC α-amylase is given in Appendix B of Novozymes' GRAS Notification GRN 000022 (1999). The Novozym 28035 α-amylase enzyme has substitutions at four additional amino acid positions as compared to the Termamyl LC α-amylase enzyme[17].

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

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Several concepts were applied in selecting the appropriate amino acid residues 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 Novozymes research.

The general structure of α-amylases is characterized by interactions of three common domains and several calcium ion binding sites[31,32]. The primary approach used to engineer an improved α-amylase was to change amino acid residues that would stabilize the domain/domain and calcium ion interactions. In addition, other amino acid substitutions were incorporated that have been shown to increase thermostability of *B. licheniformis* α-amylase[33,34].

The coding sequence for the NH_2-terminal region of the mature Novozym 28035 gene corresponds to the first thirty-five amino acids of the mature *B. amyloliquefaciens* α-amylase enzyme. The splicing of the NH_2-terminal region of the *B. amyloliquefaciens* sequence results in nine amino acid differences between Novozym 28035 and wild-type *B. licheniformis* α-amylase. The *B. amyloliquefaciens* mature enzyme is also two amino acid residues shorter at the NH_2-terminal end than the mature *B. licheniformis* enzyme. Five other amino acid changes were made in the Termamyl LC α-amylase and substitutions at an additional four amino acid positions were introduced in the Novozym 28035 α-amylase. These changes provide increased stability at low pH, low calcium concentration, and high temperature.

3.3 Sequence Comparison to other *B. licheniformis* α-amylases

The Novozym 28035 modified *B. licheniformis* α-amylase amino acid sequence has been compared to other *B. licheniformis* α-amylase amino acid sequences: Novozymes' Termamyl LC[30], Novozymes' Termamyl[35], GenBank protein accession sequence M38570[36], and GenBank nucleotide accession sequence E01158[37]. Homology among these *B. licheniformis* α-amylases is indicated in the table below:

	Novozym 28035	Termamyl LC	Termamyl	M38570	E01158
Novozym 28035	100	99.2	95.9	95.5	92.2
Termamyl LC		100	96.7	96.3	93.0
Termamyl			100	99.2	96.0
M38570				100	95.6
E01158					100

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Novozym 28035 is 95.9% identical to Termamyl, 95.5% identical to M38570 and 92.2% identical to E01158. This is within the range of similarity of the three other *B. licheniformis* α-amylases with each other: Termamyl is 99.2% identical to M38570 and 96.0% identical to E01158; M38570 is 95.6% identical to E01158. In addition, Novozym 28035 is 99.2% identical to Termamyl LC.

None of the differences among these five α-amylases are found in conserved amino acid residues identified as structurally or functionally important for α-amylases in general[31,32].

3.4 Enzymatic Activity

Novozym 28035 α-amylase is functionally equivalent to other α-amylases marketed by Novozymes'. These include wild-type *B. licheniformis* α-amylase (Termamyl) that is affirmed as GRAS as a carbohydrase in 21 CFR §184.1027, Novozymes' Termamyl LC α-amylase that is the subject of the GRAS Notification GRN 000022[30], Novozymes' Termamyl SC that is the subject of the GRAS Notification GRN 000024[38], and wild-type *B. amyloliquefaciens* α-amylase (BAN) that is affirmed as GRAS as a carbohydrase in 21 CFR §184.1148. Experiments were carried out to verify that Novozym 28035 α-amylase exhibited catalytic properties and functional effects in starch hydrolysis substantially similar to those of the other α-amylases. The experiments conducted were: monitoring changes in carbohydrate spectra during starch hydrolysis by HPLC[39] and analysis of glucose yield and oligosaccharide composition by HPLC[40]. In addition, the specific activity of Novozym 28035 and other α-amylases was measured under standard conditions (37°C, pH 7.3)[41]. These experiments demonstrate that Novozym 28035 is functionally equivalent to other marketed and GRAS α-amylases with respect to the starch hydrolytic process and products and exhibits a specific activity within in the range of naturally occurring α-amylases.

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4. MANUFACTURING PROCESS

This section describes the manufacturing process for the modified Novozym 28035 α-amylase which follows standard industry practices based on generally available and accepted methods used for the production of microbial enzymes[42-44]. The quality management system used in the manufacturing process for the Novozym 28035 α-amylase complies with the requirements of ISO 9001. Novozym 28035 is also manufactured in accordance with current Good Manufacturing Practices (cGMP).

4.1 Raw Materials

The raw materials used in the fermentation and recovery process for the Novozym 28035 α-amylase enzyme concentrate are standard ingredients used in the enzyme industry[42-44]. The raw materials conform to Food Chemicals Codex (FCC)[45] specifications except those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal specifications have been made in line with FCC requirements. On arrival at Novozymes 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 used in fermentation and recovery 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 is less than 1%.

4.2 Fermentation Process

Novozym 28035 is manufactured by submerged 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 to ensure absence of foreign microorganisms and to confirm strain identity.

4.2.1 Production Organism

Each batch of the fermentation process is initiated with a lyophilized stock culture of the production organism *B. licheniformis*, MOL2083, 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.

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4.2.2 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.

4.3.1 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 - evaporation
4. Ultrafiltration
5. Pre- and Germ Filtration - for removal of residual production strain organisms and as a general precaution against microbial degradation

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4.3.2 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.

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5. COMPOSITION AND SPECIFICATIONS

5.1 Formulation

Novozym 28035 will be available in a liquid formulation with the following typical composition:

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

5.2 Specifications

Novozym 28035 complies with the purity criteria recommended for enzyme preparations as described in FCC[45]. In addition, Novozym 28035 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 (JECFA) in Compendium of Food Additive Specifications[46].

The following specifications have been established for Novozym 28035:

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

Heavy metals, lead, arsenic, antibiotic activity, and mycotoxins are analyzed at regular intervals.

The heavy metals and lead specifications meet FCC and exceed JECFA requirements. The arsenic, total viable count and *E.coli* specifications meet JECFA requirements and are not included in FCC. The total coliforms and *Salmonella* specifications meet both FCC and JECFA. The antibiotic activity and

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mycotoxins specifications meet JECFA and are not included in FCC (although FCC mentions mycotoxins but has not established tolerances).

5.3 Absence of Production Organism in the Product

The absence of the production organism is an established specification for the commercial product, Novozym 28035. This production microorganism specification is a Novozymes' specification and is not mentioned in FCC or JECFA. The production organism does not end up in food and therefore the first step in the safety assessment as described by IFBC[4] is satisfactorily addressed.

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6. APPLICATION

6.1 Mode of Action

Novozym 28035 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, Novozym 28035 is an α-amylase used for the liquefaction of starch in the production of syrups[47-52].

In the alcohol industry, Novozym 28035 is an α-amylase used for thinning of starch in distilling mashes[47,53].

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 cGMP.

The recommended use level for Novozym 28035 is 0.35 kg per ton of starch. The optimum use level depends on the desired effect and the specific circumstances in the processing plant.

6.4 Enzyme Residues in the Final Food

Novozym 28035 is used as a processing aid and is not directly added 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)[47-50]. 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[54]. There was no detectable enzyme protein in the final syrup sample. The detection limit for the experimental assay is 1.4 ng/ml.

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7. SAFETY EVALUATION

As stated most recently by FDA[12,13], 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[1,2]. If the organism is nontoxigenic and nonpathogenic, then it is assumed that food or food ingredients produced from the organism, using cGMP, is safe to consume[4]. 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[15,55,56]. Carbohydrase 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 Novozym 28035, according to the concepts initially outlined by Pariza and Foster (1983) and further developed by IFBC[4] in 1990, the EU SCF[5] in 1991, the OECD[6] in 1992, FAO/WHO[7] in 1996, ILSI Europe Novel Food Task Force[8] in 1996, and updated by Pariza and Johnson in 2001[2] 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 introduced DNA sequences, 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.

7.1.1 Safe Strain Lineage

The genetically modified *B. licheniformis* production strain MOL2083 is considered safe because the host strain is a derivative of a well-established safe strain lineage[2], the only DNA sequences incorporated were well-characterized and strictly defined *B. licheniformis* chromosomal DNA sequences and the modified Novozym 28035 α-amylase gene, the incorporated DNA sequences

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were stably integrated in the *B. licheniformis* chromosome at three specific defined sites, and the incorporated DNA is known not to encode or express any harmful or toxic substances (see Sections 2 and 3).

The initial *B. licheniformis* host strain, SJ1707, used in the construction of the Novozym 28035 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 Novozymes has used safely for industrial production of enzymes marketed and sold since 1972, including an enzyme preparation that was the subject of the GRAS affirmation petition[14] submitted by Novozymes (formerly Novo Nordisk) and affirmed by FDA in 1983[15] which codified the regulation for carbohydrase and protease enzyme products from *B. licheniformis* as GRAS substances[57].

The *B. licheniformis* strain SJ1707, has also been used as a common host strain for several years in the construction of production strains for several other commercially marketed and sold Novozymes enzyme products including, SP844, SP703, SP501, Termamyl LC, and Termamyl SC. 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, the Termamyl SC production strain contains a modified *B. stearothermophilus* 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 enzyme for the detergent industry. The SP501 production strain is used to make cyclodextrin glycosyl transferase for the starch industry. The Termamyl LC and Termamyl SC production strains are used to make modified α-amylase enzymes for the starch and alcohol industry. Novozymes has determined that the Termamyl LC and Termamyl SC α-amylase enzyme preparations, derived from very closely related genetically modified strains of *B. licheniformis* are GRAS[30,38]. FDA indicated in its Agency Response Letters that it had no questions at the time regarding Novozymes' (formerly Novo Nordisk) GRAS determinations[58,59].

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 criteria for GILSP microorganisms[6] and meet the criteria for a safe production

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microorganism as described by Pariza and Foster (1983), Pariza and Johnson (2001), and several expert groups[3-8].

7.1.2 Toxicological tests

Toxicological testing, confirming the safety of enzyme preparations derived from the *B. licheniformis* production strains discussed in Section 7.1.1, was done as indicated in the table below. An X indicates the study was done, a - indicates the study was not done.

Test substance Batch no.	SP844 PPY 5499	SP703 PPY 5067	SP501 PPA 4357	Termamyl LC PPY 5977	Termamyl SC PPY 6347
Geno tox:					
Ames test	x	x	x	x	x
Chromosome aberration test	x	x	x	x	x
Mouse lymphoma test	-	-	x	-	-
In vivo tox:					
4-week oral in rats	x	x	-	-	-
13 week oral in rats	-	-	x	x	x
Comments	No treatment related effects	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	0.87 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.

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[60]. A summary of the toxicity data on Termamyl LC[61] was submitted to the FDA Office

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of Premarket Approval as part of the GRAS notification documentation for Termamyl LC[30].

In addition, as suggested by Pariza and Johnson (2001) for protein engineered enzymes, a limited toxicological test was done. The test article for this study was a test batch of the Novozym 28035 α-amylase enzyme preparation produced according to the description given in Section 4, except that final standardization was omitted. The test article was administered daily by oral (gavage) to rats for two consecutive weeks[62]. There were no unscheduled deaths, no treatment related clinical signs, no treatment related effects in body weight gains or food consumption, no treatment related changes in hematological or serum clinical chemistry parameters, and no organ weight changes or macroscopic changes that were considered related to treatment. Daily administration of the test article for two consecutive weeks by the oral route (gavage) to rats at low, intermediate, and high doses did not induce any signs of systemic toxicity. No treatment related effects were observed at up to 0.96 g TOS/kg bw/day. This toxicological test confirmed the safety of the Novozym 28035 production strain, MOL2083, and the modified Novozym 28035 α-amylase enzyme.

All of the *B. licheniformis* strain SJ1707 derived production strains meet the criteria for a safe production microorganism as described by several expert groups[1,2,3-8]. The outcome of the appropriate safety evaluations outlined by these expert groups supports the determination that the Novozym 28035 production strain, MOL2083, 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 Novozym 28035 α-amylase enzyme.

7.2 Safety of the α-Amylase Enzyme

Enzyme proteins themselves do not generally raise safety concerns[1,12,63]. As shown in Section 3, Novozym 28035 is an α-amylase, 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*[1,2]. In particular, bacterial α-amylases have been used in the starch industry since the 1950s[49] and, more recently, thermostable α-amylases from *B. licheniformis* and *B. stearothermophilus* have been used[47-50]. In 1973, GRAS petition 3G0026[14], which proposed the affirmation of the GRAS status of carbohydrase and protease preparations from

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B. licheniformis, was accepted for filing by the FDA[14]. Carbohydrases from *B. licheniformis* have been marketed by Novozymes 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].

Novozymes has determined that two other modified α-amylase enzyme preparations, Termamyl LC and Termamyl SC, are GRAS[30,38]. FDA indicated in its Agency Response Letters that it had no questions at the time regarding Novozymes' (formerly Novo Nordisk) GRAS determinations[6,7]. Novozymes has been commercially marketing and selling these enzymes for two years, during which time they have been safely used in the starch and alcohol industries.

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[3,6-11,64]. 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[10,65,66]. 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[9,11].

The Novozym 28035 α-amylase enzyme from the genetically modified *B. licheniformis* strain MOL2083 is substantially equivalent to other α-amylases, including unmodified *B. licheniformis* α-amylases and the modified Termamyl LC α-amylase. Novozym 28035 α-amylase is functionally equivalent and within the range of natural variation of *B. licheniformis* 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. licheniformis* exhibit slightly different properties[67,68] and have nearly, but not exactly, identical amino acid sequences[30].

The Novozym 28035 α-amylase gene has been modified to increase stability at lower calcium concentrations and lower pH than naturally occurring

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B. licheniformis α-amylases (see Section 3). However, the properties and amino acid sequence of Novozym 28035 α-amylase are within the range of naturally occurring biological diversity of *B. licheniformis* α-amylases (see Section 3). Novozym 28035 α-amylase has the same molecular weight, greater than 92% amino acid identity, and significantly similar structure to other *B. licheniformis* α-amylases. In addition, Novozym 28035 α-amylase has the same functional activity as other *B. licheniformis* α-amylases (see Section 3).

Novozymes 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[69,70]. These modified enzymes are substantially equivalent to traditional microbially derived proteases, amylases and lipases. Novozymes 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 Novozym 28035 α-amylase is substantially equivalent to other *B. licheniformis* α-amylases and is safe for use in food.

7.3 Safety of the Manufacturing Process

Novozym 28035 α-amylase meets the general and additional requirements for enzyme preparations as outlined in the monograph on Enzyme Preparations in the FCC[45]. As described in Section 4, the α-amylase preparation is produced in accordance with cGMP, 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[42-44]. The manufacturing process for Novozym 28035 α-amylase is safe.

7.4 Potential Dietary Exposure

7.4.1 Exposure from Use in Industrial Starch Processing

As stated in Section 6.4, Novozymes has experimentally verified that the Novozym 28035 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.

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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 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

Based on the exposure assessments in Sections 7.4.1 and 7.4.2, there is essentially no potential for human exposure to Novozym 28035 α-amylase by consumption of food. This lack of exposure to Novozym 28035 α-amylase when used in industrial starch processing or in the production of alcohol supports the conclusion that it is safe for its intended use.

7.5 Conclusion

The basic conclusions and guidelines for determining the safety of enzymes used in food and food processing as stated by Pariza and Foster (1983), reiterated by FDA[11], and updated by Pariza and Johnson (2001) can be applied to the Novozym 28035 α-amylase. In particular, enzyme preparations derived, using cGMPs, from a nonpathogenic and nontoxigenic microorganism, which does not produce antibiotics, would not ordinarily present a basis for a safety concern and will be safe to consume at the low levels encountered in processed foods. On the basis of a review of the published literature and unpublished information presented in the previous sections and the safety evaluation elaborated in Sections 7.1 - 7.4 supporting the safety of the production strain, the enzyme preparation, the manufacturing process, as well as a consideration of the potential dietary exposure, the Novozym 28035 α-amylase enzyme preparation produced by *B. licheniformis* expressing a gene encoding a modified *B. licheniformis* α-amylase can be safely manufactured and used as a processing aid in the starch and alcohol industry as well as in other food and non-food applications.

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End Submission