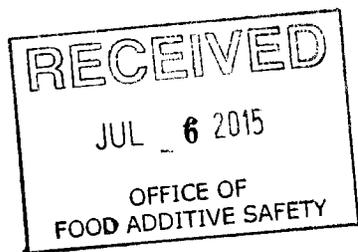


GRAS Notice (GRN) No. 587

<http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm>

ORIGINAL SUBMISSION



June 30, 2015

GRAS Notification Program
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
US Food And Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Sir or Madam,

We are hereby submitting one eCopy and one paper copy, a generally recognized as safe (GRAS) notification, in accordance with proposed 21 C.F.R. § 170.36, for Novozymes' *Acetolactate decarboxylase* (ALDC) enzyme preparation produced by a genetically modified strain of *Bacillus licheniformis*.

The electronic copy is provided on a virus-free CD, and is an exact copy of the paper submission.

Novozymes has determined through scientific procedures that ALDC is generally recognized as safe for use in the food industry as a processing aid for brewing.

Please contact me by direct telephone at [REDACTED] or email at jao@novozymes.com if you have any questions or require additional information.

Sincerely,

(b) (6)

[REDACTED]
Janet Oesterling
Regulatory Affairs Specialist III

Enclosures

Novozymes North America, Inc.
Regulatory Affairs
77 Perry Chapel Church Road, P.O. Box 576
Franklinton, North Carolina 27525

Tel: 919-494-3000

Fax: 919-494-3420

www.novozymes.com

000002

June 30, 2015

GRAS Notification Program
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
US Food And Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Sir or Madam,

We are hereby submitting one eCopy and one paper copy, a generally recognized as safe (GRAS) notification, in accordance with proposed 21 C.F.R. § 170.36, for Novozymes' *Acetolactate decarboxylase* (ALDC) enzyme preparation produced by a genetically modified strain of *Bacillus licheniformis*.

The electronic copy is provided on a virus-free CD, and is an exact copy of the paper submission.

Novozymes has determined through scientific procedures that ALDC is generally recognized as safe for use in the food industry as a processing aid for brewing.

Please contact me by direct telephone at (b) (6) or email at jao@novozymes.com if you have any questions or require additional information.

Sincerely,

(b) (6)

Janet Oesterling
Regulatory Affairs Specialist III

Enclosures

Novozymes North America, Inc.
Regulatory Affairs
77 Perry Chapel Church Road, P.O. Box 576
Franklinton, North Carolina 27525

Tel: 919-494-3000

Fax: 919-494-3420

www.novozymes.com

000003

June 30, 2015

RE: GRAS Notification - Exemption Claim

Dear Sir or Madam:

Pursuant to the proposed 21C.F.R. § 170.36 (c)(1) Novozymes North America Inc. hereby claims that *Acetolactate decarboxylase* preparation produced by submerged fermentation of a genetically modified *Bacillus licheniformis* is Generally Recognized as Safe; therefore, they are 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.*

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.*

Acetolactate decarboxylase (ALDC) preparation produced by a genetically modified *Bacillus licheniformis* production strain

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

The ALDC is used as a processing aid for conversion of alpha-acetolactate directly into acetoin to be further used as an ingredient in a variety of beer beverage products. 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 at reasonable times at Novozymes North America, Inc. or will be sent to FDA upon request.

(b) (6)



Janet Oesterling
Regulatory Affairs Specialist III

06-30-15
Date

**An Acetolactate decarboxylase preparation produced by a
genetically modified strain of
*Bacillus licheniformis***

**Janet Oesterling, Regulatory Affairs, Novozymes North America, Inc., USA
Jonathan McDonough, Regulatory Affairs, Novozymes North America, Inc., USA**

June 2015

1. GENERAL INTRODUCTION	4
2. PRODUCTION MICROORGANISM	5
2.1 Production Strain.....	5
2.2 Recipient Strain.....	5
2.3 ALDC Expression Plasmid.....	6
2.4 Construction of the recombinant microorganism.....	6
2.5 Stability of the Introduced Genetic Sequences.....	6
2.6 Antibiotic Resistance Gene	7
2.7 Absence of Production Organism in Product	7
3. MANUFACTURING PROCESS	7
3.1 Raw Materials.....	7
3.2 Fermentation Process	8
3.2.1 Production Organism	8
3.2.2 Criteria for the Rejection of Fermentation Batches	8
3.3 Recovery Process	8
3.3.1 Purification Process.....	9
3.3.2 Formulation and Standardization Processes.....	9
3.4 Quality Control of Finished Product	9
4. ENZYME IDENTITY	9
5. COMPOSITION AND SPECIFICATIONS	9
5.1 Quantitative Composition.....	10
5.2 Specifications	10
6. APPLICATION	10
6.1 Mode of Action	10
6.2 Use Levels	11
6.3 Enzyme Residues in the Final Food	11
7. SAFETY EVALUATION	12
7.1 Safety of the Production Strain	12
7.1.1 Safe Strain Lineage	13
7.2 Safety of the Donor Organism.....	14
7.3 Safety of the ALDC Enzyme	15
7.4 Safety of the Manufacturing Process.....	16
7.5 Safety studies.....	16
7.5.1 Description of Test Material	16
Novozymes / Acetolactate decarboxylase	2

7.5.2	Studies	16
7.6	Estimates of Human Consumption and Safety Margin	16
7.6.2	Safety margin	17
7.7	Results and Conclusion	18
8.	LIST OF APPENDICES	19
9.	LIST OF REFERENCES	20

1. GENERAL INTRODUCTION

The subject of this notification is an acetolactate decarboxylase (henceforth ALDC) preparation produced by submerged fermentation of a genetically modified *Bacillus licheniformis* microorganism carrying the gene from *Bacillus brevis* coding for an ALDC. ALDC converts alpha-acetolactate directly into acetoin thus avoiding the slow oxidative decarboxylation and the subsequent yeast reduction stage. Addition of ALDC to the fermentation accelerates the maturation of beer with results in quick and improved vessel utilization, thus increasing capacity.

The active enzyme is acetolactate decarboxylase (EC 4.1.1.5, CAS 9025-02-9).

The information provided in the following sections is the basis for our determination of general recognition of safety of this ALDC enzyme preparation. Our safety evaluation in Section 7 includes an evaluation of the production strain, the donor strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure to the preparation.

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food (1) (2) (Appendix 1). The production organism for this ALDC, *Bacillus licheniformis*, is discussed in Sections 2 and 7. *Bacillus licheniformis*, is generally considered to be non-pathogenic and non-toxicogenic (3) and is often mentioned as an example of a well characterized and safe production strain with a long history of safe use. It is widely recognized as a harmless contaminant found in many foods. (4)

Bacillus licheniformis has been used in the fermentation industry for the production of enzymes, antibiotics, and other specialty chemicals and was exempted from EPA review under TSCA (5). Various enzymes have been produced by *Bacillus licheniformis* and are GRAS substances (GRASP 3G0026 and 0G0363 and GRN 22, 24, 72, 79; 265, 277, 472) (6). In addition, *Bacillus licheniformis* is classified as a Risk Group 1 organism according to the National Institutes of Health Guidelines for Research Involving Recombinant Molecules. (7) Risk Group 1 organisms are those not associated with disease in healthy adult humans.

The *Bacillus licheniformis* host strain is derived from a safe strain lineage comprising production strains for more than 10 enzyme preparations which have full toxicological safety studies (i.e. 13-week oral toxicity study in rats, Ames test and chromosomal aberration test or micronucleus assay).

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 (4) (5) (8) (9) (10) (11) (12). 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.

This notification includes information that addresses the safety of the enzyme source, the enzyme component, the manufacturing process and a consideration of dietary exposure which covers all the issues relevant to a safety evaluation of an enzyme preparation. Based on critical review and evaluation of its published and unpublished information, Novozymes concludes through scientific procedures that the subject of this notification, meeting appropriate food grade specifications and produced in accordance with current good manufacturing practices, is GRAS for the intended conditions of use.

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The *Bacillus licheniformis* production strain, designated JA3876, was derived via the recipient strain, AEB1763, from a natural isolate of *Bacillus licheniformis* strain DSM 9552. 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 (9). It also meets the criteria for a safe production microorganism as described by Pariza and Foster (2) and later Pariza and Johnson (1) and several expert groups (4) (5) (8) (9) (10) (11) (12).

The *aldB*-expression plasmid, used in the strain construction, pJA3860, contains strictly defined chromosomal DNA fragments and synthetic DNA linker sequences. The DNA sequence for the introduced ALDC is based on the *aldB* encoding sequence from *Bacillus brevis*. In the *aldB* expression cassette used for the construction of the production strain, the DNA sequence encoding the mature ALDC is fused to the DNA sequence encoding the signal peptide from the amyl gene of *Bacillus licheniformis*, to enable efficient secretion.

2.2 Recipient Strain

The recipient strain AEB1763 used in the construction of the ALDC production strain was modified at several chromosomal loci during strain development to inactivate genes encoding a number of proteases. Also deletion of a gene essential for sporulation was performed, eliminating the ability to sporulate, together with the deletion of additional genes encoding unwanted proteins that can be present in the culture supernatant. The lack of these represents improvements in the product purity, safety and stability.

2.3 ALDC Expression Plasmid

The expression plasmid, pJA3860, used to transform the *Bacillus licheniformis* recipient strain AEB1763 is based on the well-known *Bacillus* vectors pE194 (13) and pUB110 (14) from *Staphylococcus aureus*. No elements of these vectors are left in the production strain. The plasmid contains the expression cassette consisting of a fragment of a *Bacillus* promoter with promoter elements from *B. licheniformis*, *B. amyloliquefaciens* and *B. thurigiensis*, the *aldB* coding sequence and a transcriptional terminator.

Following the terminator, a non-coding DNA sequence is inserted to enable targeted integration of the transforming DNA into the genome of the recipient strain. Only the expression cassette with elements between the promoter fragment and the terminator are present in the final production strain. This has been confirmed by Southern blot analysis and PCR analysis followed by DNA sequencing.

2.4 Construction of the Recombinant Microorganism

The production strain, *Bacillus licheniformis* JA3876, was constructed from the recipient strain AEB1763 through the following steps:

- 1) Plasmid pJA3860 was integrated into five specific loci in strain AEB1763 by targeted homologous recombination to these loci using a two-step integration approach. Targeted integration of the expression cassettes at these loci allows the expression of the ALDC gene *aldB* from the promoter.
- 2) The resulting ALDC strain containing one copy of the *aldB* gene at each of the five target loci was named JA3876.

Sequence confirmation of the inserted expression cassettes and the flanking regions at each of the integration loci was performed in the production strain.

2.5 Stability of the Introduced Genetic Sequences

The genetic stability of the introduced DNA sequences was determined by Southern hybridization. Analysis of samples from end of production using an *aldB* gene specific probe showed an identical band pattern compared to the reference production strain (JA3876), demonstrating the genetic stability of the introduced DNA during production. The transforming DNA is stably integrated into the *Bacillus licheniformis* chromosome and, as such, is poorly mobilized for genetic transfer to other organisms and is mitotically stable.

2.6 Antibiotic Resistance Gene

No functional antibiotic resistance genes were left in the strain as a result of the genetic modifications. The absence of these genes was verified by genome sequence analysis.

2.7 Absence of Production Organism in Product

The absence of the production organism is an established specification for the commercial product. 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.

3. MANUFACTURING PROCESS

This section describes the manufacturing process for the ALDC which follows standard industry practices (15) (16) (17). The quality management system used in the manufacturing process for the ALDC complies with the requirements of ISO 9001. It is produced under a standard manufacturing process as outlined by Aunstrup (16) and in accordance with current Good Manufacturing Practices, using ingredients that are accepted for general use in foods, and under conditions that ensure a controlled fermentation. The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the Food Chemicals Codex (18). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (19).

3.1 Raw Materials

The raw materials used in the fermentation and recovery process for the ALDC enzyme concentrate are standard ingredients used in the enzyme industry (15) (16) (17). The raw materials conform to Food Chemicals Codex 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 in the product is less than 1%.

3.2 Fermentation Process

The ALDC is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *Bacillus 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 done to ensure absence of foreign microorganisms and confirm strain identity.

3.2.1 Production Organism

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

3.2.2 Criteria for the Rejection of Fermentation Batches

Growth characteristics during fermentation are observed both macroscopically and microscopically. Samples are taken from both the seed fermenter and the main fermenter 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.

3.3 Recovery Process

The recovery process is a multi-step operation designed to separate the desired enzyme from the microbial biomass and partially purify, concentrate, and stabilize the enzyme.

3.3.1 Purification Process

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

1. Pretreatment - pH adjustment and flocculation
2. Primary Separation – vacuum drum filtration or centrifugation
3. Concentration - ultrafiltration and/or evaporation
4. Pre- and Germ Filtration - for removal of residual production strain organisms and as a general precaution against microbial degradation
5. Preservation and Stabilization of the liquid enzyme concentrate
6. Final concentration – evaporation and/or ultrafiltration if enzyme concentration is too low to reach target yield

3.3.2 Formulation and Standardization Processes

The enzyme concentrate is stabilized with glycerol. The liquid product is formulated by preservation with potassium sorbate and standardized by addition of water.

3.4 Quality Control of Finished Product

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

4. ENZYME IDENTITY

Key enzyme and protein chemical characteristics of the ALDC are given below:

Classification	Acetolactate decarboxylase
IUBMB nomenclature:	Acetolactate decarboxylase
EC No.:	4.1.1.5
CAS No.:	9025-02-9
Specificity:	converts acetolactate directly into acetoin
Amino acid sequence:	the total nucleotide and amino acid sequences have been determined

5. COMPOSITION AND SPECIFICATIONS

The ALDC enzyme concentration is presently available in a liquid formulation for use in brewing applications.

5.1 Quantitative Composition

The ALDC preparation has the following typical composition:

Enzyme Solids (TOS ^a)	Approx.	5 %
Glycerol (stabilizer)	Approx.	56 %
Water (diluent)	Approx.	38.8%
Potassium sorbate	Approx.	0.2 %

^a=Total Organic Solids, define as: 100% - water – ash – diluents.

5.2 Specifications

The ALDC enzyme preparation complies with the recommended purity criteria for enzyme preparations as described in *Food Chemicals Codex* (18). In addition, it 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 Specifications¹⁵.

This is demonstrated by analytical test results of the enzyme preparations batches in Table 1 below.

Table 1. Analytical data for three food enzyme batches.

Parameter	Specifications	PPD36805	PPD36816	PPD36770
ALDC activity	ADU(L)/g	27500	25800	24200
Total viable count	Upper limit 50,000	100	<100	100
Lead	Not more than 5 mg/kg	ND	ND	ND
Salmonella sp.	Absent in 25 g of sample	ND	ND	ND
Total coliforms	Not more than 30 per gr	< 10	< 10	< 10
Escherichia coli	Absent in 25 g of sample	ND	ND	ND
Antimicrobial activity	Not detected	ND	ND	ND

6. APPLICATION

6.1 Mode of Action

The active enzyme is acetolactate decarboxylase (EC 4.1.1.5), commonly known as ALDC. Acetolactate decarboxylase catalyses the decarboxylation of alpha-acetolactate to acetoin thus avoiding the slow oxidative decarboxylation and the subsequent yeast reduction stage allowing for quick and improved vessel utilization.

The ALDC preparation is used as a processing aid during the food manufacturing. The typical food processes where this food enzyme is used include brewing processes.

The benefits of the action of the food enzyme in brewing beverage processes and beverage alcohol (distilling) processes are:

- Reduced formation of diacetyl during fermentation and thereby a reduction of the off-flavours caused by this substance
- A faster maturation process and thereby a shorter production time, e.g. to meet peak season capacity demands

The acetolactate decarboxylase converts alpha-acetolactate directly into acetoin, which thereby reduces the amount of diacetyl formed during fermentation and maturation, which consequently reduces the needed length of the maturation process.

6.2 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 conditions of use of the ALDC preparation during food processing do not only depend on the type of application, but also on the food production process of each individual food manufacturer. In order to ensure optimal effectiveness of the enzyme at an acceptable economic cost, the dosage, reaction time, process conditions and processing steps are adjusted.

The recommended use level for the food enzyme in brewing processes is up to 50 ADU(L)/liter of fermenting wort. This corresponds to 0.133 mg/TOS.

6.3 Enzyme Residues in the Final Food

The ALDC food enzyme catalyzes the decarboxylation of alpha-acetoacetate. It is used in food manufacturing as a processing aid in order to ease, optimize and ensure consistent processes, and to minimize potential end product variations.

When using the food enzyme in brewing beverage processes the enzyme will not be functional in the final beer, either because there is no substrate (alpha-acetoacetate) for the enzyme as it has been depleted, or because the enzyme has been removed and/or denatured in the process. Therefore, the enzyme does not exert a function in the final beer.

In the case of beverage alcohol (distilling) processes the substrate will be depleted by the end of the maturation period, wherefore the enzyme will not be functional in the final fermented wort. The fermented wort is distilled, and any food enzyme (active and inactive) will remain in the stillage; accordingly, the distilled beverage alcohol is free of food enzyme.

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

The safety of the production organism must be the prime consideration in assessing the degree of safety of an enzyme preparation intended for use in food (2) (1). If the organism is non-toxigenic and non-pathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, is safe to consume (20). Pariza and Foster (2) define a non-toxigenic organism as “one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure” and a 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 (21) (3). *B. licheniformis* has a long history of safe industrial use for the production of enzymes used in human food. It is widely recognized as a harmless contaminant found in many foods (20). *B. licheniformis* has been used in the fermentation industry for the production of enzymes, antibiotics, and other specialty chemicals. Various enzymes are produced by *B. licheniformis* and are considered GRAS substances (α -amylases – GRASP 3G0026 and 0G0363 and GRN 22, 24, 79; pullulanase – GRN 72; and xylanase – GRN 472) (6). In addition, *B. licheniformis* is classified as a Risk Group 1 organism according to the National Institutes of Health Guidelines for Research Involving Recombinant Molecules. Risk Group 1 organisms are those not associated with disease in healthy adult humans.

The *B. licheniformis* host strain is derived from a safe strain lineage comprising production strains for nine enzyme preparations which have full toxicological safety studies (i.e. 13 week oral toxicity study in rats, Ames test and chromosomal aberration test or micronucleus assay), cf. section 7.1.1 below.

An evaluation of the genetically modified production microorganism for the ALDC, embodying the concepts initially outlined by Pariza and Foster, 1983 (2) and further developed by IFBC in 1990 (20), the EU SCF in 1991 (8), the OECD in 1992 (9), ILSI Europe Novel Food Task Force in 1996 (12), FAO/WHO in 1996 (11), JECFA in 1998 (19) and Pariza and Johnson in 2001 (1) 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 incorporated DNA, 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 Section 2 and 3.

The genetic modifications are well characterized and specific utilizing well-known plasmids for the vector constructs, and the introduced genetic material does not encode and express any known harmful or toxic substances. The production strain is considered a safe strain for the production of the acetolactate decarboxylase enzyme.

7.1.1 Safe Strain Lineage

The safety of this *B. licheniformis* production strain was established following published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients (1) (4). The host strain, *Bacillus licheniformis* PP2982, has been thoroughly characterized as shown in sections 2.2. The introduced DNA is well-known and characterized in section 2.3 and 2.4. The procedures used to modify the host organism are well defined and commonly used. Therefore, the elements needed to establish a safe strain lineage as defined in Pariza and Johnson, 2001 (1) have been met.

Novozymes' used the decision tree (Appendix 3) in Pariza and Johnson 2001 (1) as a basis for our safety assessment. The production strain is genetically modified by rDNA techniques as discussed in section 2. The expressed enzyme product is an ALDC. The enzyme preparation is free of DNA encoding transferable antibiotic resistance gene DNA. The introduced DNA is well characterized and safe for the construction of microorganisms to be used in the production of food grade products. The DNA is stably integrated into the chromosome at five specific sites in the chromosome and the incorporated DNA is known not to encode or express any harmful or toxic substances.

Novozymes has extensive experience working with *Bacillus licheniformis* production strains and has developed expertise in identifying and characterizing these strains in order to prevent contamination and ensure continuing acceptable, economic yields of a functional enzyme product. Research scientists, fermentation engineers, chemical operators, and quality control technicians follow standard aseptic microbiological procedures as well as specific Novozymes protocols for monitoring the biological activity, growth, and physiological characteristics of the production organism during strain improvement programs and during large scale industrial fermentations. In addition, the final commercial enzyme product must perform reproducibly, meet Novozymes' technical service department requirements, and consistently meet the needs of customers in the food industry. All of these periodic and continuous monitoring activities serve not only to guarantee customer satisfaction with Novozymes' enzyme products but also indicate that no unexpected secondary effects of the genetic modifications have been observed. Furthermore the information included in this GRN has been reviewed by Novozymes Regulatory Affairs staff for suitability.

Finally, the production strain is derived from a safe lineage. Novozymes has used *B. licheniformis* production strains for over 20 years. An overview of some of the Novozymes products produced by *Bacillus licheniformis* strains and the safety studies conducted on those products is shown below (Table 2).

Table 2

Enzyme	EC No.	Predecessor strain ¹	Donor strain	Safety studies ²
Alpha-amylase (GRASP 0G0363)	3.2.1.1	<i>Bacillus licheniformis</i> Si3	<i>Bacillus stearothermophilus</i>	Yes
Alpha-amylase (GRN 22)	3.2.1.1	<i>Bacillus licheniformis</i> SJ1707	<i>Bacillus licheniformis</i>	Yes
Cyclodextrin glucanotransferase	2.4.1.19	<i>Bacillus licheniformis</i> SJ1707	<i>Thermoanaerobacter sp.</i>	Yes
Alpha-amylase	3.2.1.1	<i>Bacillus licheniformis</i> SJ1707	<i>Bacillus licheniformis</i>	Yes
Alpha-amylase	3.2.1.1	<i>Bacillus licheniformis</i> SJ1904	<i>Bacillus licheniformis</i>	Yes
Alpha-amylase	3.2.1.1	<i>Bacillus licheniformis</i> MDT223	<i>Bacillus stearothermophilus</i>	Yes
Alpha-amylase	3.2.1.1	<i>Bacillus licheniformis</i> MDT223	<i>Bacillus amyloliquefaciens</i>	Yes
Serine protease	3.4.21.1	<i>Bacillus licheniformis</i> MDT223	<i>Nocardiopsis prasina</i>	Yes
Alpha-amylase	3.2.1.1	<i>Bacillus licheniformis</i> MDT223	<i>Bacillus licheniformis</i>	Yes
Xylanase	3.2.1.8	<i>Bacillus licheniformis</i> MDT223	<i>Bacillus licheniformis</i>	Yes

Table 2. Novozymes products derived from *B. licheniformis* strains ¹The predecessor strain shows strains in the GM construction pathway that are in common with the host strain AEB1763 lineage. ²At least the following: *in vitro* test for gene mutations in bacteria (Ames); *in vitro* test for chromosomal aberration or *in vitro* micronucleus assay; 13 week sub chronic oral toxicity study in rats.

As shown (Table 2), safety studies have been performed for the same enzyme in different strains in the lineage, supporting the fact that the genetic modifications performed in the *B. licheniformis* strain lineage of the host do not result in safety concerns. Additionally, no safety issues are observed when different products that are produced in the same strain (e.g., amylases and xylanases) are investigated, demonstrating that the safety of the strains in the lineage is not product-dependent.

Novozymes has repeatedly used the procedures outlined by Pariza and Johnson 2001 (1) to evaluate the enzymes derived from *B. licheniformis* production strains. Therefore, following the evaluation outlined in this section this production strain is considered to be derived from a safe lineage and is safe for use in the production of enzyme preparations for use in food.

7.2 Safety of the Donor Organism

As noted above, it is the safety of the production strain that should be the primary concern when assessing the safety of an enzyme used for food.

The donor organism of the ALDC is *Bacillus brevis*. As indicated in Section 2 the introduced DNA is well defined and characterized. Only well characterized DNA fragments, limited solely to the ALDC coding sequence from the donor strain, are used in the construction of the genetically modified strain. The introduced DNA does not code for any known harmful or toxic substances.

7.3 Safety of the ALDC Enzyme

A wide variety of enzymes are used in food processing (2) (1). Enzyme proteins do not generally raise safety concerns (1) (2) Acetolactate decarboxylases have been used safely in food production for decades and are widespread in nature being produced by a number of prokaryotic micro-organisms, including several *Bacillus* and *Lactobacillus* species (22). Pariza and Foster (2) note that very few toxic agents have enzymatic properties. The safety of the ALDC was assessed using the Pariza and Johnson, (2001) decision tree (Appendix 3).

7.3.1 Consideration of the Allergenic Potential of the ALDC Enzyme

The ingestion of a food enzyme protein is not considered a concern for food allergy. This is based on the following considerations:

- Enzymes have a long history of safe use in food, with no indication of adverse effects or reactions.
- The majority of proteins are not food allergens. A wide variety of enzyme classes and structures are naturally present in plant and animal based foods, and based on previous experience, food enzymes are not homologues to known allergens, which make it very unlikely that a new enzyme would be a food allergen.
- Enzymes in foods are always added in concentrations in the low range of part per millions. The enzyme is typically removed or denatured during food processing, and denatured protein has been shown to be very susceptible to digestion in the gastro-intestinal system. Moreover, a wide range of naturally occurring food enzymes have been shown to be very labile in the gastro-intestinal system even in the native unprocessed form.

The above statements are further supported by the publication: "Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry" (Bindslev-Jensen *et al*, 2006) (23). The investigation comprised enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and Protein Engineered variants and comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. It was concluded from this study that ingestion of food enzymes in general is not likely to be a concern with regard to food allergy.

In order to further evaluate the possibility that the ALDC will cross-react with known allergens and induce a reaction in an already sensitized individual, a sequence homology to known food allergens was assessed. Following the guidelines developed by FAO/WHO, 2001 (24) and modified by Codex Alimentarius Commission, 2009 (25) the ALDC was compared to allergens from the FARRP

allergen protein database (<http://allergenonline.org>) as well as the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee (<http://www.allergen.org>).

A search for more than 35% identity in the amino acid sequence of the expressed protein using a window of 80 amino acids and a suitable gap penalty showed no matches. Alignment of the ALDC to each of the allergens and identity of hits with more than 35% identity over the full length of the alignment was analyzed. No significant homology was found between the ALDC and any of the allergens from the databases mentioned above. Also, a search for 100% identity over 8 contiguous amino acids was completed. Again, no significant homology was found. Consequently, oral intake of the ALDC is not anticipated to pose any food allergenic concern.

7.4 Safety of the Manufacturing Process

The ALDC enzyme preparation meets the purity criteria for enzyme preparations as outlined in the monograph on Enzyme Preparations in the *Food Chemicals Codex*. As described in Section 3, the enzyme 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 production of microbial enzymes (15) (16) (17).

7.5 Safety studies

This section describes the studies and analysis performed to evaluate the safety of the use of the ALDC.

7.5.1 Description of Test Material

The following studies were performed on test batch PPD36621 with favourable results:

- 13 Week Oral Toxicity, daily dosing, rats
- Reverse Mutation Assay (Ames test)
- *in vitro* micronucleus assay

These tests are described in Appendix 4.

7.6 Estimates of Human Consumption and Safety Margin

In the production of beer it is assumed that the totality of the food enzyme used during production will end up in the final beer.

The assumption regarding the food enzyme in the final beer is highly exaggerated since the enzyme protein and the other substances resulting from the fermentation are diluted or removed in certain processing steps. Furthermore, beer and beer-like beverages produced with the enzyme are not always produced with the maximum recommended dosage.

In order to demonstrate a worst case calculation, an exaggerated human intake for beer and beer-like beverages was used and is based on NHANES Survey 2003-2006 (26). The highest mean daily level of intake of beer is for men between the ages of 21 and 64. On average, the highest consumption of beer in men is 455 grams per day.

According to the CDC Anthropometric Reference Data (27) the average weight of an adult female over the age of 20 is 75.4 kilos. This is the lowest weight average between male and female. Using the highest mean intake of beer and the lowest weight average represents the “worst case” scenario. Based on this, 6.03 grams of beer and beer-like beverage is consumed per kg/bw/day.

Therefore, the safety margin calculation derived from this method is highly exaggerated.

The ALDC has an average activity of 25833 ADU/g and an approximate content of 6.9% TOS (Total Organic Substances from the fermentation, mainly protein and carbohydrate components).

This corresponds to an activity/TOS ratio of:

$$25833 \div 0.069 \div 1000 = 374.4 \text{ ADU/mg TOS}$$

This will result in an exposure of:

$$50 \text{ ADU (dosage)} \div 374.4 \text{ (mg/TOS ratio)} \times 6.03 \text{ (grams of beer consumed)} \div 1000 = 0.000805 \text{ mg TOS/kg bw/day.}$$

Therefore the Total Maximum Daily Intake (TMDI) of the food enzyme by consumers is:

$$0.000805 \text{ mg/TOS/kg bw/day.}$$

The TMDI contribution from beverage alcohol is assumed to be zero due to the distilling process.

7.6.2 Safety margin

The safety margin is calculated as dose level with no adverse effect (NOAEL) divided by the estimated human consumption. The NOAEL dose level in the 13 weeks oral toxicity study in rats conducted on ALDC, PPD36621 was the highest dosage possible, 1018 mg TOS/kg bw/day. (see Appendix 4)

NOAEL (mg TOS/kg bw/day)	1018
TMDI (Brewing beverage processes), (mg TOS/kg bw/day)	0.000805
Safety margin	1.26×10^6

The safety margin can thus be calculated to be $1018/0.000805$ or approximately 1.26×10^6 .

7.7 Results and Conclusion

On the basis of the evaluation contained in Section 7, a review of the published literature, the history of safe use of *B. licheniformis*, and the limited and well defined nature of the genetic modifications, the ALDC enzyme preparation can be safely manufactured and used as a food enzyme to reduce the “maturation time” of beer by accelerating the conversion of alpha-acetolactate directly into acetoin.

8. LIST OF APPENDICES

1. Pariza, M.W. and Johnson, E.A.. Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century. Reg. Tox and Pharm 33: 173-186, 2001.
2. de Boer AS, Priest F, Diderichsen B. On the Industrial Use of *Bacillus licheniformis*: a review. Appl. Microbiol. Biotechnol. 40, 595-598, 1994.
3. Pariza and Johnson Decision Tree Analysis
4. Summary of Toxicity Data. ALDC PPD36621. 28 August 2015, File No. 2015-14510-02. LUNA No. 2015-14510-02.

9. List of References

1. Pariza, M.W. and Johnson, E.A.. Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century. *Reg. Tox and Pharm* 33: 173-186, 2001.
2. Pariza, M.W. and Foster, E.M.. Determining the Safety of Enzymes Used in Food Processing. *Journal of Food Protection*, 46:5:453-468, 1983.
3. de Boer AS, Priest F, Diderichsen B. On the Industrial Use of *Bacillus licheniformis*: a review. *Appl. Microbiol. Biotechnol.* 40, 595-598, 1994.
4. IFBC (International Food Biotechnology Council). Chapter 4: Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms in Biotechnologies and Food: Assuring the Safety of Foods Produced by Genetic Modification. 1990, Vol. 12, pp. S1-S196.
5. Environmental Protection Agency (EPA). Biotechnology Program Under Toxic Substances Control Act (TSCA) *Bacillus licheniformis* Final Risk Assessment 1997. (<http://epa.gov/oppt/biotech/pubs/fra/fra005.htm>).
6. GRAS Notice Inventory: <http://www.accessdata.fda.gov>.
7. Guidelines for Research Involving Recombinant DNA Molecules. Federal Register, Dec.19, 2001 (66 FR 57970).
8. EU Scientific Committee for Food. Guidelines for the presentation of data on food enzymes. Reports of the Scientific Committee for Food, 27th series, 1991.
9. Organisation for Economic Cooperation and Development, Safety Evaluation of Foods Derived by Modern Biotechnology, 1993.
10. Berkowitz, D. and Maryanski, J.. Implications of biotechnology on international food standards and codes of practice. Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission, Eighteenth Session, Geneva, July 3-12, 1989.
11. FAO/WHO. Biotechnology and Food Safety, Report of a Joint FAO/WHO Consultation. FAO Food and Nutrition Paper 61. Rome, Italy. 1996.
12. Jonas, D.A., et al. The Safety Assessment of Novel Foods, Guidelines prepared by ILSI Europe Novel Food Task Force, *Food Chemical Toxicology*, Vol. 34, 1996. pp. 931-940.

13. Horinouchi, S., and Weisblum, B.. Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide and streptogramin type-b antibiotics. *J. Bacteriol.*, 150, 804-814, 1982.
14. Gryczan, T.J., Contente, S., Dubnau, D. : Characterization of *Staphylococcus aureus* plasmids introduced by transformation into *Bacillus subtilis*. *J. Bact.* 134, 318-329 (1978).
15. Kirk, O., Damhus, T., Borchert, T. V., Fuglsang, C. C., Olsen, H. S., Hansen, T. T., et al. *Enzyme Applications, Industrial*. Kirk-Othmer Encyclopedia of Chemical Technology, Vol. 9, pp 566-620, 2000.
16. Aunstrup, K.. *Production, Isolation, and Economics of Extracellular Enzymes in Applied Biochemistry and Bioengineering, Volume 2, Enzyme Technology*, Eds. Wingard, L.B., Katchalski-Katzir, E. And Goldstein, L, pp. 28-68, 1979.
17. Aunstrup, K., Andersen, O., Falch, E.A., and Nielsen, T.K.. *Production of Microbial Enzymes in Microbial Technology*, 2nd ed., Vol. I, Eds. Pepler, H.J. and Perlman, D., Chapter 9, pp.282-309, 1979.
18. United States Pharmacopeial Convention. *Food Chemical Codex. Edition 9. Monograph: Enzyme Preparations*. United States Pharmacopeial Convention, Board of Trustees, 2014. Pg 410-415.
19. JECFA (Joint FAO/WHO Expert Committee on Food Additives. *General Specifications and Considerations for Enzyme Preparations Used in Food Processing. Compendium of Food Additive Specifications*, FAO FNP (Food and Nutrition Paper) 52, Add. 9, FAO, Rome 2001 and FAO JECFA Monographs 3 (2006).
20. IFBC (International Food Biotechnology Council). Chapter 4: *Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms in Biotechnologies and Food: Assuring the Safety of Foods Produced by Genetic Modification*. 1990, Vol. 12, pp. S1-S196.
21. Olempska-Bier, Z.S. , Merker, R.I, Ditto, Mary D., and DiNovi, M.J. *Food-processing enzymes from recombinant microorganisms—a review*. *Reg. Tox and Pharm* 45:144-158, 2006.
22. Godtfredsen SE, Lorck H, Sigsgaard P (1983) On the occurrence of alpha-acetolactate decarboxylases among microorganisms. *Carlsberg Res. Commun.* 48, 239-247.
23. Bindslev-Jensen C, Skov PS, Roggen EL, Hvass P, Brinch DS *Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry*. *Food Chem. Toxicol.* 44, 1909-1915, 2006.

24. Evaluation of Allergenicity of Genetically Modified Foods (Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology 22-25 January 2001), Food and Agriculture Organization of the United Nations (FAO), Rome, 2001.
25. Codex Alimentarius commission. Foods derived from modern biotechnology. FAO/WHO, Rome, pp.1-85, 2009.
26. Alcoholic Beverage Consumption by Adults 21 Years and Over in the United States: Results from the National Health and Nutrition Examination Survey, 2003-2006.
27. Center for Disease Control, Anthropometric Reference Data for Children and Adults: United States, 2007-2010.
28. Food and Drug Administration. Enzyme Preparations from animal and plant sources; Affirmation of GRAS status as direct food ingredients. Fed. Regist. 60, 1995. pp. 32904-32912.

Pages 000027-000044 have been removed in accordance with copyright laws. The references removed are:

Pariza, M.W. and Johnson, E.A. Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century. *Reg. Tox and Pharm* 33: 173-186, 2001.

de Boer AS, Priest F, Diderichsen B. On the Industrial Use of *Bacillus licheniformis*: a review. *Appl. Microbiol. Biotechnol.* 40, 595-598, 1994.

Appendix 3- Pariza & Johnson Decision Tree analysis of an *Acetolactate decarboxylase* (ALDC) produced by a genetically modified strain of *Bacillus licheniformis*.

This *Acetolactate decarboxylase* produced by a genetically modified strain of *Bacillus licheniformis* was evaluated according to the decision tree published in Pariza and Johnson, 2001. The result of the evaluation is presented below.

Decision Tree

1. Is the production strain genetically modified?

YES

If yes, go to 2.

The production strain *Bacillus licheniformis* designated JA3876, was derived via the recipient AEB1763 from a natural isolate.

2. Is the production strain modified using rDNA techniques?

YES

If yes, go to 3.

3. Issues relating to the introduced DNA are addressed in 3a-3e.

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

YES

Enzymes, including ALDC, have a long history of use in food and animal feed. *Acetolactate decarboxylases* have been used safely in food production for decades and are widespread in nature being produced by a number of prokaryotic micro-organisms, including several *Bacillus* and *Lactobacillus* species. The ALDC, which is the subject of this GRN, is produced by a production strain that is from a safe strain lineage with an extended history of safe use

If yes go to 3c. If no, go to 3b

- b. Is the NOAEL for the test article in the appropriate short-term oral studies sufficiently high to ensure safety? (Not required since 3a is YES however the answer here is also **YES**)
- c. Is the test article free of transferable antibiotic resistance gene DNA?

YES

No functional antibiotic resistance genes were left in the strain as a result of the genetic modifications. The absence of these genes was verified.

If yes go to 3e. If no go to 3d.

- e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food products?

YES

The genetic modifications are well characterized and specific and the incorporated DNA does not encode and express any known harmful or toxic substances.

If yes, go to 4.

4. Is the introduced DNA randomly integrated into the chromosome?

NO

Site specific integration of the DNA was achieved at five selected loci of the *B. licheniformis* chromosome. Sequence confirmation was performed in the production strain.

If yes go to 5. If no, go to 6.

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

YES

If yes go to 6. If no go to 7.

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?

YES

The production strain is a *B.licheniformis* derived via the recipient strain AEB1763, which is from a natural isolate. Novozymes has safely used *B. licheniformis* production strains derived from this isolate for over 20 years including an enzyme preparation that is the subject of the GRAS affirmation petition 3G0026 submitted by Novo Nordisk and affirmed by FDA in 1983.

If yes the test article is ACCEPTED.

Toxicology
Toxicology & Product Safety

Date: 28 August 2015
File: 2015-14510-02
Ref.: SGE/TrGQ

SUMMARY OF TOXICITY DATA

ALDC, batch PPD36621
from *Bacillus licheniformis*

Author:
Trine Østergaard Lund
Signe Elvig-Jørgensen

Issued by:
Novozymes A/S
Krogshøjvej 36
DK-2880 Bagsvaerd
Denmark

CONTENTS

	PAGE
1. ABSTRACT	3
2. TEST SUBSTANCE	3
2.1 Characterization	3
3. MUTAGENICITY	4
3.1 Bacterial Reverse Mutation assay (Ames test)	4
3.2 <i>In vitro</i> Micronucleus assay	4
4. GENERAL TOXICITY	5
4.1 13 Week Oral Toxicity Study in Rats	5
5. REFERENCES	6
5.1 Study Reports	6
LAST PAGE	6

1. ABSTRACT

The below series of toxicological studies were undertaken to evaluate the safety of ALDC, batch PPD36621.

The studies were carried out in accordance with current OECD guidelines. All studies were performed in compliance with the principles of Good Laboratory Practice (GLP). The studies were performed at HLS (UK), Covance Laboratories Ltd. (UK) and Novozymes A/S (DK).

The main conclusions of these studies can be summarized as follows:

- ALDC, batch PPD36621 did not induce gene mutations in the Ames test, neither in the presence nor absence of S-9 mix.
- ALDC, batch PPD36621 did not cause an increase in the induction of micronuclei in cultured human lymphocytes in this *in vitro* micronucleus test using human lymphocytes, neither in the presence nor in the absence of S-9 mix.
- In a 13 week oral gavage study in rats, ALDC, batch PPD36621 was generally well tolerated up to the dose level of 100% (corresponding to 1018mg TOS/kg bw/day). This dose level was therefore considered to be the NOAEL of the study.

Based on the present toxicity data it can be concluded that ALDC, batch PPD36621 exhibit no toxicological effects under the experimental conditions described.

2. TEST SUBSTANCE

ALDC is a liquid enzyme concentrate containing an acetolactate decarboxylase. ALDC, batch PPD36621 is produced in a strain belonging to a safe strain lineage of *Bacillus licheniformis*.

2.1 Characterization

ALDC, batch PPD36621 was used for the conduct of all the toxicological studies. The characterization of the toxbatch is presented in Table 1.

Table1. Characterization Data of ALDC, batch PPD36621

Batch number	PPD36621
Activity	22000 ADU(L)/g
Water (KF) (% w/w)	88.7
Dry matter (% w/w)	11.3
Ash (% w/w)	1.5
Total Organic Solids (TOS ¹) (% w/w)	9.8

¹ % TOS is calculated as 100% - % water - % ash - % diluents.

3. MUTAGENICITY

3.1 Bacterial Reverse Mutation assay (Ames test)

ALDC, batch PPD36621 was examined for mutagenic activity in the bacterial reverse mutation assay (Ames test) using *Salmonella typhimurium* strain TA1535, TA100, TA1537, TA98 and *Escherichia coli* WP2uvrApKM101.

The study was conducted in accordance with the general recommendations in OECD Guideline for testing of chemicals, No. 471: Bacterial Reverse Mutation Assay” (July 1997) concerning the general specifications of the test. However, the exposure of test bacteria in liquid culture (“treat and plate”) is not specifically described in this guideline.

Crude enzyme preparations, like the present test substance contain the free amino acids histidine and tryptophan, most often in an amount, which exceeds the critical concentration for incorporation in the direct standard assay.

To overcome this problem, all strains were exposed to the test substance in liquid culture (“treat and plate assay”). Bacteria were exposed to six doses of the test substance in a phosphate buffered nutrient broth for 3 hours with 5 mg TOS per mL as the highest concentration. After incubation the test substance was removed by centrifugation prior to plating.

The study was conducted with and without the metabolic activation system S9 mix: a liver preparation from male rats, pre-treated with Aroclor 1254 (S9) added the co-factors required for mixed function oxidase activity. All results were confirmed by conducting two complete and independent experiments.

The test substance contains an abundance of various nutrients, and composes a rich growth medium to the test bacteria. These circumstances are reflected to an extent in the present study. Weak and insignificant growth stimulation as well as inhibition are present in some test series. No distinct toxicity was observed.

No treatments of any of the *Salmonella* and *E. coli* strains with the test substance resulted in any increases in the number of revertant colonies that meet the criteria for a positive or equivocal response.

Based on the results obtained in this study, it is concluded that ALDC, batch PPD36621 did not show any evidence of mutagenic activity when tested under the conditions applied in this bacterial reverse mutation test.

3.2 *In vitro* Micronucleus assay

In order to assess the clastogenic and the aneugenic activity of ALDC, batch PPD36621, its effects on the frequency of micronuclei was investigated in cultured human peripheral blood lymphocytes applying the cytokinesis-block methodology.

The study was conducted according to GLP, in compliance with the OECD test guideline 487 (adopted in 2010).

Heparinized whole blood cultures, pooled from two female donors, were established, and division of the lymphocytes was stimulated by adding phytohaemagglutinin (PHA) to the cultures.

Three different treatment schedules with duplicate cultures were performed. Cultures were

exposed to the test substance, negative control (vehicle), or appropriate positive controls for 3 hours in the presence and absence of metabolic activation (S-9 mix) and harvested 24 hours after the beginning of treatment (3+21 hour treatment). Additionally, a continuous 24-hour treatment without S-9 mix was included with harvesting 24 hours after removal of the test substance (24+24 hour treatment). Treatments with the test substance covered a broad range of doses, separated by narrow intervals. The highest concentration used was 5000 µg TOS/mL, an acceptable maximum concentration for *in vitro* micronucleus studies according to current regulatory guidelines.

The cultures were treated with cytochalasin-B after removal of the test substance. Three concentrations, covering an appropriate range of cytotoxicity, were selected for scoring of micronuclei by evaluating the effect of the test substance on the replication Index (RI). 2000 cells per concentration (1000 cells from each replicate culture) were scored.

The proportion of binucleate cells with micronuclei in all cultures of the vehicle controls (purified water) was within the limits of the historical ranges. The positive controls induced statistically significant increases in the proportion of cells with micronuclei, thus demonstrating the sensitivity of the test procedure and the metabolic activity of the S-9 mix employed.

Treatment of the cells with the test substance resulted in frequencies of micronucleated binuclear cells (MNBN cells), which were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle controls and which fell within normal ranges for the majority of concentrations analyzed. Exceptions to this were seen at the intermediate dose levels after the 3+21 hour -S-9 treatment and the 24+24 hour treatment, where small statistically increases were apparent. However, these increases were small with one or both replicate cultures exhibiting MNBN cell values that fell within the normal ranges. Therefore, these increases were considered spurious and of no biological importance.

It was concluded that ALDC, batch PPD36621 did not induce micronuclei in cultured human peripheral blood lymphocytes either in the absence or presence of S-9 mix under the experimental conditions employed for this study.

4. GENERAL TOXICITY

4.1 13 Week Oral Gavage Toxicity Study in Rats

The objective of this study was to assess the systemic toxic potential of Acetolactate decarboxylase (ALDC), batch PPD36621 when administered orally by gavage to Sprague-Dawley rats for 13 weeks.

The study was conducted according to GLP, in compliance with the OECD test guideline 408 (adopted in 1998).

Three groups, each comprising 10 males and 10 females, received doses of 10, 33 or 100% of the Acetolactate decarboxylase batch. A similarly constituted control group received the vehicle (reverse osmosis water) at the same volume dose (10 mL/kg body weight).

During the study, clinical condition, detailed physical and arena observations, sensory reactivity, grip strength, motor activity, body weight, food consumption, water consumption (by visual assessment), ophthalmoscopy, haematology (peripheral blood), blood chemistry, organ weight, macropathology and histopathology investigations were undertaken.

General appearance and behavior, sensory reactivity responses, grip strength and

motor activity were not affected by treatment, there were no deaths during the treatment period and there was no effect of treatment on bodyweight gain or on food and water consumption. There were no treatment-related ophthalmic findings. The haematology and blood chemistry investigations during Week 13 did not identify any toxicologically significant differences from controls. Analysis of organ weights for animals killed after 13 weeks of treatment revealed slightly high body weight-adjusted kidney and epididymides weights for males given 100% ALDC. There were no treatment-related macroscopic or histopathological findings.

It is concluded that oral administration of Acetolactate decarboxylase (ALDC), batch PPD36621, to Sprague-Dawley rats at doses up to 100% of the ALDC batch (equivalent to 1.018 gTOS/kg/day) for 13 weeks was well-tolerated and did not cause any adverse change. The no-observed adverse-effect level (NOAEL) was considered to be 100% of the ALDC batch (equivalent to 1.018 gTOS/kg/day).

5. REFERENCES

Study reports

Novozymes A/S: Study No. 20148004: Characterization of GMM ALDC (acetolactate decarboxylase) Toxbatch PPD36621 from *Bacillus licheniformis*. (February 2015). LUNA file:2014-04173.

Novozymes A/S: Study No.: 20148049. ALDC, batch PPD36621: Test for Mutagenic Acitivity with Strains of *Salmonella typhimurium* and *Escherichia coli*. (January 2015). LUNA file: 2014-18420.

Covance Laboratories: Study No.: 8306994. Novozymes Reference No.: 20146046: ALDC, batch PPD36621: Induction of micronuclei in cultured human peripheral blood lymphocytes. (January 2015). LUNA file: 2015-00449.

Huntingdon Life Sciences: Study LKG0103. Novozymes Reference no.:20156009: ALDC, batch PPD36621: Toxicity Study by Oral Gavage Administration to Sprague-Dawley Rats for 13 Weeks.

SUBMISSION END