GR

ORIGINAL SUBMISSION

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June 5, 2006

Robert Martin, Ph.D.
Deputy Division Director
Division of Biotech and GRAS Notice Review
Office of Food Additive Safety (HFS-255)
Center for Food Safety and Applied Nutrition
Food And Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

REC'D JUN - 7 2005

Dear Dr. Martin,

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' pullulanase enzyme preparation produced by *Bacillus subtilis* expressing the gene encoding a pullulanase from *Bacillus acidopullulyticus*. The enzyme preparation is intended for use in the brewing industry.

Please contact me by direct telephone at 919 494-3151, direct fax at 919 494-3420 or email at lobg@novozymes.com if you have any questions or require additional information.

Sincerely,

Lori Gregg
Regulatory Specialist

Enclosures (3 binders)

Novozymes North America, Inc.
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June 5, 2006

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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 pullulanase preparations produced by submerged fermentation of *Bacillus subtilis* expressing the gene encoding a pullulanase from *Bacillus acidopullulyticus* are 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

REC'D JUN - 7 2005

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

Pullulanase preparation produced by *Bacillus subtilis* expressing the gene encoding a pullulanase from *Bacillus acidopullulyticus.*

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

The enzyme preparation is intended for use in the brewing industry as a processing aid. 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.

June 5, 2006

John Carroll
Director, Regulatory Affairs

Date

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A pullulanase preparation produced by *Bacillus subtilis* expressing the gene encoding a pullulanase from *Bacillus acidopullulyticus*

Lori Gregg, Regulatory Affairs, Novozymes North America, Inc., USA
Kim Rygaard Nielsen, Regulatory Affairs, Novozymes A/S, Denmark

June 2006

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1. GENERAL INTRODUCTION

The subject of this notification is a pullulanase preparation produced by submerged fermentation of a *Bacillus subtilis* microorganism carrying the gene coding for a pullulanase from *Bacillus acidopullulyticus*. The Novozymes A/S trade name used for the pullulanase preparation is Novozym® 26062.

The enzyme is a pullulanase (EC 3.2.1.41), which catalyzes the hydrolysis of 1,6-alpha-glucosidic linkages in amylopectin. It is therefore well suited for "debranching" starch after liquefaction.

Novozym 26062 is used in the brewing industry as a processing aid in the saccharification of liquefied starch. Use of a pullulanase enzyme in addition to glucoamylase enzymes will speed up the hydrolysis of branched dextrins and will increase the level of fermentable sugars in the wort.

The information provided in the following sections is the basis for our determination of general recognition of safety of this pullulanase enzyme preparation produced by *B. subtilis* expressing a gene encoding a pullulanase from *B. acidopullulyticus*. Our safety evaluation in Section 7 includes an evaluation of the production strain including an evaluation of safe strain lineage, 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]. *Bacillus subtilis* is generally considered to be non-pathogenic and non-toxigenic and is often mentioned as an example of a well characterized and safe production strain with a long history of safe use. The production organism for this pullulanase, *B. subtilis*, is discussed in Sections 2 and 7.

The production organism is derived from the *Bacillus subtilis* A164 host strain that has been used for over 15 years by Novozymes in the development of strains to be used for manufacturing enzymes used as processing aids in the food industry. In section 7.1.1, we show the basis for a safe strain lineage for this production strain following the procedure outlined in Pariza and Johnson 2001 (Appendix 1).

The evaluation of the safety of the *Bacillus subtilis* host organism is very important since all, but a small fraction of the DNA, is that of the recipient host organism. 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[3-9]. The genetic modifications used to construct the production microorganism are well defined and are described in Section 2. The recipient *B. subtilis* host strain, A164Δ5, has been used as a host strain in the construction of production strains for four other Novozymes enzyme products. For each of these production strains the *B. subtilis* host strain, A164Δ5, was used as a

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. subtilis* host strain chromosome by homologous recombination. All of these production strains comply with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) micro-organisms[10] and meet the criteria for a safe production micro-organism as described by Pariza and Foster[2], Pariza and Johnson[1] and several expert groups[3-9]. Toxicological testing, including 13 week oral toxicity in rats, Ames test, and human lymphocyte cytogenetic assay, confirming the safety of enzyme preparations derived from these four *B. subtilis* production strains, was done. No toxicological effects were observed for any of the test substances produced by strains derived from the *B. subtilis* host strain, A164Δ5.

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The *B. subtilis* strain, designated MDT96, was constructed by plasmid transformation of the recipient strain, designated A164Δ5 (see Section 2.2). 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[10]. It also meets the criteria for a safe production microorganism as described by Pariza and Foster[2] and several expert groups[1,3-9].

The pullulanase expression cassette (Figure 1) used in the strain construction contains strictly defined bacterial chromosomal DNA fragments and synthetic DNA linker sequences. The specific DNA sequences include: a gene encoding a *B. acidopullulyticus* pullulanase enzyme; a triple tandom promoter comprising the well known strong amyL promoter variant denoted PamyL4199[11], the consensus version of the promoter of the *B. amyloliquefaciens* α-amylase gene, amyQ[12]; the *B. thuringiensis* cryIIIA promoter[13,14]; the cryIIIA mRNA stabilizer[14,15]; the *Bacillus clausii* alkaline protease gene (aprH) ribosome binding site and transcription terminator[16].

2.2 Recipient Organism

The recipient strain is *Bacillus subtilis* strain A164 *spoIIAC*, *nprE*, *aprE*, *amyE*, *srfC* (*B. subtilis* A164Δ5). The parent strain, *B. subtilis* A164, is identical to strain ATCC 6051a, which is identical to or a derivative of American Type Culture Collection (ATCC) 6051, the type strain for *B. subtilis.*

B. subtilis A164 was genetically modified by replacement of five resident genes with deleted versions. These genes comprise those encoding sigma F, neutral protease, alkaline protease, amylase, and surfactin C (designated *spoIIAC*, *nprE*, *aprE*, *amyE*, and *srfC*). The resulting strain is therefore non-sporulating, protease deficient, amylase negative, and surfactin negative. Deletion of these genes results in a higher yielding more efficient production strain for the pullulanase enzyme.

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The host strain *B. subtilis* A164Δ5 was constructed from A164 by sequential deletion of five genes. In each case, a temperature-sensitive plasmid (able to replicate at the permissive temperature of 34°C but not at the non-permissive temperature of 45°C) bearing a deleted version of the gene was used to replace the native version of the gene on the *B. subtilis* chromosome with the deleted version. The temperature-sensitive plasmid was introduced into the host by conjugation, inserted into the chromosomal copy of the gene via homologous recombination by selective growth at the non-permissive temperature, and then excised from the chromosome and lost from the cell by non-selective growth at the permissive temperature. Strains in which the chromosomal gene was replaced by the deleted version were identified by corresponding characteristics and genetic tests.

1) The *spoIIAC* gene (encoding sporulation sigma factor F) was deleted from the chromosome of A164 using a temperature-sensitive plasmid that bears a deleted version of the *spoIIA* gene. The resulting strain, A164 *spoIIAC*Δ, is unable to sporulate.

2) The *nprE* gene (encoding neutral metalloprotease) was deleted from the chromosome of A164 *spoIIAC*Δ using a temperature-sensitive plasmid that bears a deleted version of the *nprE* gene. The resulting strain, A164Δ2, produces a smaller zone of clearing (relative to strain A164 *spoIIAC*Δ) on agar containing non-fat dry milk, due to a reduction in protease production.

3) The *aprE* gene (encoding alkaline protease) was deleted from the chromosome of A164Δ2 using a temperature-sensitive plasmid that bears a deleted version of the *aprE* gene. The resulting strain, A164Δ3, produces a very small zone of clearing on agar containing non-fat dry milk, due to elimination of the two major extracellular proteases.

4) The *amyE* gene (encoding alpha-amylase) was deleted from the chromosome of A164Δ3 using a temperature-sensitive plasmid that bears a deleted version of the *amyE* gene. The resulting strain, A164Δ4, produces no zone of clearing on agar containing starch-azure, due to the elimination of amylase production.

5) The *srfC* gene (encoding an enzyme involved in synthesis of surfactin) was deleted from the chromosome of A164Δ4 using a temperature-sensitive plasmid that bears a deleted version of the *srfC* gene. The resulting strain, A164Δ5, is unable to produce surfactin.

2.3 Introduced DNA

The pullulanase (pulC) expression cassette transferred to the *Bacillus subtilis* host strain, A164Δ5 by homologous recombination in a single copy at the *B. subtilis* α-amylase gene (amyE) chromosomal locus is shown below.

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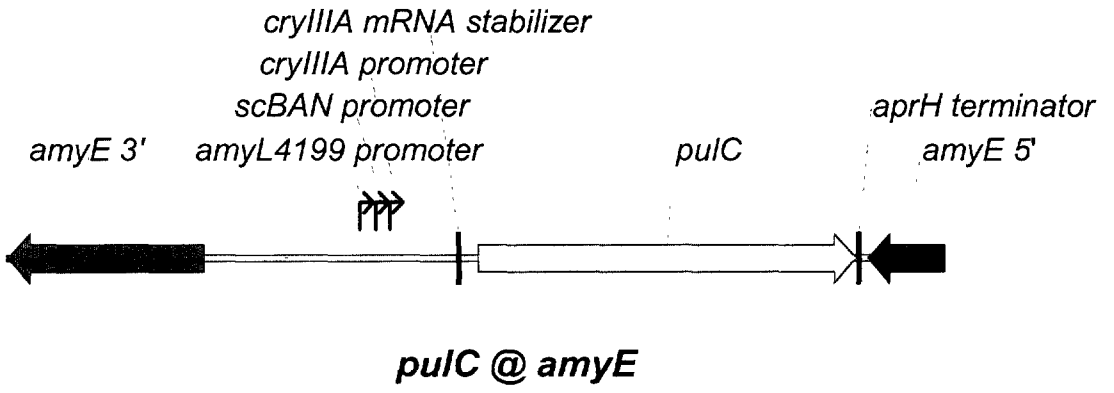

Figure 1. Schematic drawing of the pullulanase pulC expression cassette

2.4 Construction of the Production strain MDT96

As a result of the double crossover integration event at the amyE locus, only the expression cassette shown above and a chloramphenicol (*cat*) resistance marker from the expression plasmid integrate into the strain. The *cat* gene is then deleted by a Cambelling in and Campbelling out (also known as two-step gene replacement)[17] transformation event with a plasmid containing a deleted version of the *cat* gene. As a result, the final integrated strain, MDT96, is free of any antibiotic resistance genes.

2.5 Stability of the Introduced Genetic Sequences

The presence of the introduced DNA sequences was determined by Southern hybridization to assess the stability and potential for transfer of genetic material as a component of the safety evaluation of the production microorganism[3-9]. The transforming DNA is stably integrated into the *B. subtilis* chromosome and, as such, is poorly mobilizable for genetic transfer to other organisms and is mitotically stable[10].

2.6 Antibiotic Resistance Gene

The introduced DNA does not contain any coding antibiotic resistance genes.

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[3] is satisfactorily addressed.

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3. MANUFACTURING PROCESS

This section describes the manufacturing process for the pullulanase which follows standard industry practices[18-20]. The quality management system used in the manufacturing process for the pullulanase complies with the requirements of ISO 9001. It is also manufactured in accordance with current good manufacturing practices.

3.1 Raw Materials

The raw materials used in the fermentation and recovery process for the pullulanase enzyme concentrate are standard ingredients used in the enzyme industry[18-20]. 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 pullulanase is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *B.subtilis* 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 lyophilized stock culture of the production organism, *B. subtilis,* 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

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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.

3.3 Recovery Process

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

3.3.1 Purification Process

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

- Pretreatment and flocculation
- Filtrations - vacuum drum filtration, polish filtrations and germ filtration
- Concentration - ultrafiltration and/or evaporation
- Preservation and Stabilization of the liquid enzyme concentrate.

3.3.2 Formulation and Standardization Processes

The stabilized concentrate is blended with water, sucrose/glucose and preserved with sodium benzoate and potassium sorbate. The product is standardized according to the product specification.

3.4 Quality Control of Finished Product

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

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4. ENZYME IDENTITY

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

Classification	Pullulanase (generic name)
IUB nomenclature:	α-Dextrin endo-1,6-α-glucosidase
IUB No.:	EC 3.2.1.41
CAS No.:	9075-68-7
Specificity:	Pullulanase hydrolyzes 1,6-α-D-glucosidic linkages in pullulan, amylopectin and glycogen, and in the α- and β-amylase limit dextrins of amylopectin and glycogen.
Amino acid sequence:	the total nucleotide and amino acid sequences have been determined

5. COMPOSITION AND SPECIFICATIONS

The pullulanase enzyme preparation is presently available in a formula for use in brewing applications. The Novozymes A/S trade name used for the pullulanase preparation is

5.1 Quantitative Composition

Novozym 26062 has the following typical composition:

Enzyme solids (TOS)	approx.	2 %
Water	approx.	57 %
Sucrose/glucose	approx.	41 %
Sodium benzoate		0.1 %
Potassium sorbate		0.3 %

Novozym 26062 has a typical activity of 400 PUN-RPA/g (Pullulanase Unit Novo). Endo-pullulanase hydrolyzes the *α*-1,6-glycosidic bonds in red pullulan, releasing red substrate degradation products. The amount of colour released is measured spectrophotometrically and is proportional to the endo-pullulanase activity in the sample. The activity is measured relative to a Novozymes pullulanase standard.

5.2 Specifications

The pullulanase enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in *Food Chemicals Codex*[21]. In addition, the pullulanase preparation conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications[9].

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6. APPLICATION

6.1 Mode of Action

The active enzyme in is a pullulanase (EC 3.2.1.41), which hydrolyzes 1,6-α-D-glucosidic linkages in pullulan, amylopectin and glycogen, and in the α- and β-amylase limit dextrins of amylopectin and glycogen.

is used in the brewing industry as a processing aid in the saccharification of liquefied starch. Use of a pullulanase enzyme in addition to glucoamylase enzymes will speed up the hydrolysis of branched dextrins and will increase the level of fermentable sugars in the wort. The enzyme may be added during mashing or after mashing. After the post mashing saccharification process, the wort is usually boiled before fermentation. Boiling will inactivate the enzyme.

6.2 Use Levels

The use levels of the enzyme are according to the requirements for normal production (GMP). The optimum dosage depends on the desired effect and the specific circumstances in the processing plant. However, the recommended dosage of is:

1 - 25 liters per ton of starch dry substance.

6.3 Enzyme Residues in the Final Food

The enzyme is used in the early stages of production in the brewer's mash. It is added to hydrolyse *α*- and *β*- limit dextrins with production of additional fermentable sugars. The sugars are fermented to produce alcohol. The enzyme will be inactivated during wort boiling and the protein will be removed in subsequent processing steps.

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

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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 current Good Manufacturing Practices, is safe to consume[3]. Pariza and Foster (1983)[2] 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. subtilis* meets these criteria for nontoxigenicity and nonpathogenicity.

B. subtilis as a type species is not considered to be a pathogen[22]. The species in general is often represented as an example of non-pathogenic micro-organisms[23]. Because *B. subtilis* meets the US Environmental Protection Agency (EPA) criteria for nontoxigenicity and nonpathogenicity, it is one of 10 host organisms eligible for Tier I exemption under the EPA regulations[24].

B. subtilis is widely distributed in nature, is a common contaminant in foods eaten by both man and animals, and has a history of safe use in food enzyme manufacturing[25-27]. Carbohydrases and proteases from *B. subtilis* are affirmed as GRAS by the US FDA and are covered in the regulations under 21 CFR 184.1148 and 21 CFR 184.1150.

The host organism used for cloning the pullulanase enzyme was derived from *B. subtilis*, which is a well-known and widely used microorganism in recombinant DNA research. The *Bacillus subtilis* host strain, A164Δ5, has been used as a host strain for the construction of Novozymes production strains for several years. *The* (US) National Institute of Health has exempted sporulation-deficient strains of *B. subtilis* from its Guidelines for Research involving Recombinant Molecules because it considers *B. subtilis* to be harmless.

An evaluation of the genetically modified production microorganism for the pullulanase, embodying the concepts initially outlined by Pariza and Foster, 1983 and further developed by IFBC in 1990, the EU SCF in 1991, the OECD in 1992, ILSI Europe Novel Food Task Force in 1996, FAO/WHO in 1996, JECFA in 1998 and Pariza and Johnson, 2001 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.

Because the genetic modifications are well characterized and specific, and the incorporated DNA does not encode and express any known harmful or toxic substances, the pullulanase enzyme preparation derived from the genetically modified *B.subtilis* is considered safe[3,28].

A published paper "On the safety of *Bacillus subtilis* and *B. amyloliquefaciens*: a review" is enclosed in Appendix 2.

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7.1.1 Safe Strain Lineage

The safety of this *B. subtilis* production strain was established following published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients[1,3]. The host strain, *B.subtilis* A164Δ5, has been thoroughly characterized as shown in sections 2.2. The introduced DNA is well-known and characterized in section 2.3. 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 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 a pullulanase (See section 7.3). The enzyme preparation is free of transferable coding 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 integrated into the *α*-amylase locus of the chromosome, and the production strain is well characterized by qualified scientists and technicians. Novozymes has extensive experience working with *B. subtilis* 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.

Finally, the production strain is derived from a safe lineage. Novozymes has used *B. subtilis* production strains for over 15 years. Safety studies including 13 week oral toxicity in rats, Ames test, and human lymphocyte cytogenetic assay, have been performed on enzyme preparations from these production strains. No toxicological effects were ever observed for any of the test substances produced by these *B. subtilis* production strains.

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The recipient host strain for this pullulanase, *B. subtilis* A164Δ5, is the same strain that was used in the construction of the Novozymes' production strain for another pullulanase producing *B. subtilis* strain as well as 3 other Novozyme production strains. Safety studies including 13 week oral toxicity in rats, Ames test, and human lymphocyte cytogenetic assay, were performed on the other pullulanase preparation

(Appendix 3) as well as the three other products. These studies support the view, that strains derived from *B. subtilis* A164Δ5 can be used safely for the production of food enzymes. The same well known procedures have been used to construct this *B. subtilis* production strain. Therefore, we would not expect to see any negative results in similar toxicological studies.

For the pullulanase that is the subject of this notification, we have conducted an Ames test, and an In vitro cytotoxicity test (as discussed in section 7.5). The conclusion of these tests is that the test preparation is considered non-cytotoxic and does not induce gene mutations in bacteria under the conditions of the study.

Novozymes has used the procedures outlined by Pariza and Johnson 2001[1] to evaluate the enzymes derived from *B. subtilis* 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

The donor for the pullulanase enzyme is *Bacillus acidopullulyticus*. *Bacillus acidopullulyticus* is not considered pathogenic or toxigenic. A comprehensive toxicological test program has been made on a pullulanase preparation from *Bacillus acidopullulyticus*[29], including subchronic studies, Ames test, and *in-vivo* cytogenecity test. No toxicological adverse effect was observed. In addition, the pathogenic potential of the strain in mice and rats was investigated. It was concluded that the *Bacillus acidopullulyticus* strain is avirulent.

However, the pathogenic and toxigenic potential of the particular strain of *Bacillus acidopullulyticus* used as donor for the pullulanase gene should not be of particular importance to the safety of our pullulanase enzyme preparation[3]. Pariza and Johnson[1] confirm that the safety of the production strain is the primary concern in evaluating enzyme safety. The primary issue in evaluating the safety of a production strain is its toxigenic potential. It is important to note that the only *Bacillus acidopullulyticus* DNA introduced into the *Bacillus subtilis* production organism is the protein coding sequence from *Bacillus acidopullulyticus* pullulanase gene contained in the pullulanase expression plasmid. The steps used during strain construction ensure that the incorporated *Bacillus acidopullulyticus* DNA is limited solely to the pullulanase coding sequence. Therefore, following the concepts outlined in Pariza and Johnson (2001)[1], we can conclude that all new DNA introduced into the *B. subtilis* production strain is safe and we have not changed the strain's toxigenic potential.

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7.3 Safety of the Pullulanase Enzyme

A wide variety of enzymes are used in food processing[1]. Enzyme proteins do not generally raise safety concerns[2,22,30]. Exceptions could include enzymes that produce substances that are not ordinarily digested and metabolized or that produce toxic substances[31]. Pariza and Foster[2] note that very few toxic agents have enzymatic properties. As indicated in section 4, the subject of this GRAS notification is a pullulanase, EC 3.2.1.41.

Pullulanase enzyme preparations produced by *Bacillus acidopullulyticus* are the subject of GRAS Petition 4G0295 submitted to FDA in 1984 and filed April 10, 1985. Pullulanase enzyme preparations produced by *Bacillus acidopullulyticus* have been marketed as GRAS in the US since that time. Pullulanases from genetically modified production organisms have also been marketed in the US[32]. Pullulanase enzyme preparations are the subject of GRAS Notifications No. GRN 000020 and GRN 000072. Enzymes including pullulanases have a long history of safe use as processing aids in the food industry[1].

7.3.1 Consideration of the Allergenic Potential of the Enzyme

Enzymes are proteins and proteins can be potential allergens. However, only a small percentage of all dietary proteins are allergens. Novozymes is not aware of any allergic reactions caused by the ingestion of pullulanase. Novozymes' believes that, in general, the risk of allergenicity due to ingestion of enzymes is negligible based on the following considerations:

- the long history of safe use of a wide variety of enzymes in food and food processing
- the ubiquitous nature of enzymes
- the extremely low exposure potential
- the purpose

As discussed in Section 7.3, enzyme proteins do not generally raise safety concerns. A wide variety of enzymes have been used safely for centuries in food processing. Enzymes are found in many cells and tissues of plants and animals including those that are consumed by man. They are ubiquitous and in this respect, common and ordinary. They are a normal part of the human diet.

Enzymes are a special type of protein with a highly specific catalytic function. They are essential to many biochemical reactions in microorganisms, plants, animals and humans. Due to the specific nature of enzymes, only very small amounts are required to achieve the desired effect in food. In this case the exposure to the pullulanase enzyme will be negligible due to the nature of the brewing process.

Based on the fact that:

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- there are no known cases of allergic responses to this enzyme in food,
- known cases of allergic responses to food enzymes are very rare, and
- exposure to this enzyme protein is extremely low,

the allergy risk due to the ingestion of this pullulanase is negligible.

7.4 Safety of the Manufacturing Process

The enzyme preparation meets the purity criteria for enzyme preparations as outlined in the monograph on Enzyme Preparations in the *Food Chemicals Codex*[21]. 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[18-20].

7.5 Safety studies

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

7.5.1 Description of Test Material

The safety studies described below were conducted on a liquid pullulanase enzyme concentrate that was prepared according to the description given in Section 3 except that stabilization and standardization were omitted.

7.5.2 Studies

The following studies were performed:

-Test for mutagenic activity (Ames Test)
- *In vitro* Cytotoxicity Test: Neutral Red Uptake in L929 Monolayer Culture

These tests are described in Appendix 4. The conclusion of these tests is that the test preparation is considered non-cytotoxic and does not induce gene mutations in bacteria under the conditions of the study.

7.6 Estimates of Human Consumption and Safety Margin

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As stated in section 6, [redacted] is used in the Brewing industry as a processing aid during beer production. The enzyme activity is largely removed and

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heat inactivated during the beer production process. However, in order to illustrate a "worst case" situation, the following calculation is made assuming that all enzyme activity is retained in the beer.

has a typical activity of 400 PUN-RPA/g and an approximate content of 2% TOS (Total Organic Substances from the fermentation, mainly protein and carbohydrate components).

7.6.1 Estimates of human consumption

The average human intake of beer is estimated using well-established statistics from various countries.

Per capita consumption of beer per year:

Denmark:	128.2 liter
France:	40.0 liter
United Kingdom:	102.6 liter

(Ref.: Chemical Economics Handbook. Source: Panorama of EU industry, 1994, European Commission, 1994).

USA: 81.8 liter
(Ref.: USDA. 2003 ERS/USDA Data: Food consumption (per capita) data system. US Department of Agriculture. http://www.ers.usda.gov/data/foodconsumption/spreadsheets)

In order to demonstrate a worst case calculation, an exaggerated human intake is estimated using the following assumptions.

a) The calculation is assuming that all Total Organic (TOS) originating from remains in the Beer. contains 2% TOS.

b) It is assumed that all beer is produced using as a processing aid during the production process, and that it is used at highest recommended dosage.

Figures used in the calculation, based on the highest of the above mentioned per capita intake:

Consumption of beer per person per day (128.2 litre per year)	0.351 liter/day
350 kg adjunct is used for production of	6660 liter beer
Max. Novozym 26062 dosage (Section 6.2): 25 kg per ton adjunct	25 g/kg
Enzyme Total Organic Solid per g product (Section 5.1): 2 %	0.02 g TOS/g
Weight average person:	60 kg

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"Worst case" daily intake per person of TOS from beer:

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Daily intake · Kg adjunct · Dosage · Enzyme TOS /liter beer yield =
(0.351 litre/day · 350 kg · 25 g/kg · 0.02 g TOS/g)/6660 litre = $9.22 \cdot 10^{-3}$ g TOS per day.

For an average person weighing 60 kg this corresponds to:

$1.5 \cdot 10^{-4}$ g TOS per kg body weight per day

It must, however, be emphasised that these figures are over-estimated because:
- Not all beer produced uses [redacted] as processing aid.
- The enzyme will be partly inactivated during wort boiling.
- The enzyme protein will be removed in purification steps.
- We have used the average consumption of beer for a person from Denmark which is higher than the consumption of beer per person in the US.

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 toxicity study in rats for a similar enzyme preparations from the *B. subtilis* strain (host A164Δ5) mentioned in section 7.1.1, was the highest dosages possible, approximately 1.3 g TOS per kg body weight per day (Appendix 3).

Safety margin: 1.3 g TOS/kg/day/$1.5 \cdot 10^{-4}$ g TOS/kg/day = **$8.7 \cdot 10^{3}$**.

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. subtilis,* and the limited and well defined nature of the genetic modifications, the pullulanase enzyme preparation can be safely manufactured and used as a processing aid in the brewing industry as well as in other food or non-food applications.

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Appendix 1

Pages 000026 - 000039 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Appendix 2

Pages 000041 - 000044 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Appendix 3

Enzyme Business Toxicology

Date: 2000-08-22
File: 2000-07060-02
Ref.: PBjP/JFCa/DSc

SUMMARY OF TOXICITY DATA

Pullulanase from *Bacillus deramificans* expressed in *Bacillus subtilis*

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ABSTRACT

Pullulanase (Batch No. PPY 6454) is a liquid enzyme concentrate. The main activity is a heat stable debranching 6-glucano-hydrolase. It is produced by submerged fermentation of a strain of *Bacillus subtilis*, containing the pullulanase gene code originating from *Bacillus deramificans.*

Toxicological investigations were undertaken to evaluate the safety of this Pullulanase with respect to general toxicity and mutagenicity. The main conclusions can be summarised as follows:

Oral administration to rats of Pullulanase up to 10 ml/kg body weight/day (~ 1.285 g TOS/kg/day) for 13 weeks has not revealed any significant toxic effects attributable to the test substance.

Pullulanase has shown no mutagenic activity. It did not induce gene mutations in bacteria or chromosome aberrations in cultured human blood lymphocytes.

It is overall concluded that Pullulanase can be generally recognized as safe.

1. INTRODUCTION

In order to register Pullulanase for use in the food industry, toxicological studies were undertaken to evaluate the safety of this product. Pullulanase was investigated in respect of general toxicity and mutagenicity. The results of these studies are summarized in this paper.

The studies were carried out at Huntingdon Life Sciences (Huntingdon, Cambridgeshire, England), Covance (Harrogate, North Yorkshire, England), Scantox Biology Laboratory (Ejby, Lille Skensved, Denmark) and Enzyme Business, Novo Nordisk A/S (Bagsvaerd, Denmark) during the period March 1999 to June 2000.

All studies were carried out in accordance with current guidelines of the Organisation for Economic Cooperation and Development (OECD) and they were conducted in compliance with the principles of Good Laboratory Practice (OECD, as revised in 1997).

2. TEST SUBSTANCE

2.1 Characterisation

The Pullulanase, batch PPY 6454, applied in the toxicological investigations is a mixture of three separate fermentation sub-batches. They were fermented and recovered according to the same procedures as are used for the production of commercial Pullulanase preparations. The product complies with the Food and Agriculture Organization/World Health organization (FAO/WHO), Joint Expert Committee on Food Additives (JECFA, 1996) and Food Chemical Codex (FCC, 1996) recommended purity specifications for food grade enzymes.

The principal enzyme is a 6-glucano-hydrolase catalysing the hydrolysis of the 1,6-alpha-linkages in amylopectin (E.C. # 3.2.1.41) and the preparation appears as a brown liquid. The specific density of 1.080 g/ml and the molecular weight of the enzyme protein is 105 kD. It is completely miscible with water. The enzyme activity is measured as endo-pullulanase activity, NPUN (New Pullulanase Unit Novo), with a colorimetric method based on hydrolysis of α-1.6-glycosidic bonds in red pullulan, releasing red substrate degradation products.

Pullulanase, batch PPY 6454, has been analysed for chemical and microbial content; and all the analytical results were within the set limits.

The enzyme activity was measured to be 3950 NPUN/g with an amount of 11.9% Total Organic Solids (TOS = 100% - % water - % ash). The pH was measured to 4.9.

Analyses of the antibiotic activity and microbial contamination showed that this batch complies with the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 1992) and Food Chemical Codex (FCC IV, 1996).

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2.2 Production microorganism

Pullulanase is produced by a strain of *Bacillus subtilis*, containing the pullulanase gene code originating from *Bacillus deramificans.* This genetically modified production strain meets the criteria for a safe production micro-organism. It is constructed by common transformation procedures using well-known plasmid vectors with strictly defined and well-characterized DNA sequences that are not known to encode or express any harmful or toxic substances. The development of the production strain was evaluated at every step to assess incorporation of the desired functional genetic information and to ensure no unintended sequences were incorporated. Further this production strain does not contain any resistance markers.

Bacillus subtilis is generally regarded as non-pathogenic and non-toxigenic. It has a long history of safe use and it has been used for many years in the production of enzymes, and in the last decade as recombinant organism for production of a variety of bio-industrial products.

The test article does not contain this production strain, in accordance with the product specifications.

3. STUDY SUMMARIES

3.1 General toxicity

3.1.1 13-weeks Subchronic oral toxicity in rat

Scantox Biology Laboratory, Report No: 34916. Novo Nordisk Study No.: 996013. Pullulanase, batch PPY 6454: A 13-weeks oral (gavage) toxicity study in rats.

The study was performed in accordance with the OECD guideline 408, (1998).

Groups of ten male and ten female Mol:SPRD rats received Pullulanase orally, by gavage at dosages of 1.0, 3.0 or 10.0 ml/kg/day (equivalent to 0.1285 – 0.387 – 1.285 g TOS/kg/day) daily for 13 weeks. A similarly constituted group received the vehicle alone (purified water, obtained by osmosis) and served to generate contemporaneous control data. A constant dose volume of 10 ml per kg bodyweight was used throughout the study.

No unscheduled deaths occurred. No treatment-related clinical signs were observed, neither at the twice, daily checks nor during the once per week detailed clinical observations or at the stimulus-induced reaction tests during week 11 of treatment.

No treatment-related changes in body weight, body weights gain, food consumption or at the ophthalmoscopic examinations were seen.

Treatment with Pullulanase batch 6454 had no adverse effects on parameters of haematology, clinical chemistry or urinalysis.

A dose dependent increase of urinary specific gravity was observed in males (significant for the medium and high dose group). The toxicological significance of this observation as a single entity is questionable, because of the homeostatic role, played by the kidney in water balance and because no other kidney-related findings were observed in this study.

No treatment related changes in organ weights, macroscopic or microscopic pathology were seen.

It is concluded that the oral administration to Mol:SPRD rats of Pullulanase, PPY 6454 at dosages of up to 10.0 ml/kg/day (equivalent to 1.285 g TOS/kg/day) for 13 weeks was not associated with any toxicological significant effect and that this dosage represents the No-Observed-Effect-Level (NOEL) in this study.

3.2 Mutagenicity

3.2.1 Assessment of gene mutations in bacteria

Novo Nordisk A/S Report No.: 2000-024427-01. NN Study No.: 20008003. Pullulanase (Batch No. PPY 6454): Test for mutagenic activity with strains of Salmonella typhimurium and Escherichia coli.

Pullulanase was examined in order to determine the ability to induce gene mutations in strains of *Salmonella typhimurium* and *Escherichia coli.*

The study was conducted in accordance with OECD Guideline for Testing of Chemicals No. 471 (1997), and the requirements of the Annex to European Commission Directive 92/69/EEC. However, the exposure of the test bacteria in liquid culture ("treat and plate"), as applied in this study with strains of Salmonella, is not specifically described in any guidelines.

Four histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and one tryptophan-requiring strain of *Escherichia coli* (WP2 uvrA) were applied in this study.

Crude enzyme preparations contain free amino acids such as histidine and tryptophan. Pullulanase (batch No. PPY 6454) significantly support growth of the histidine-requiring *S.typhimurium* strains by direct incorporation of the test substance on selective agar media. In order to avoid the risk of artefacts due to growth stimulation, a "treat and plate" assay was applied. Bacterial cultures were exposed to Pullulanase, solvent and appropriate positive controls in phosphate buffered nutrient broth for three hours at 37°C. After this period, all nutrients originating from the test substance and broth were removed by centrifugation of the bacterial suspensions.

The growth stimulation of the tryptophan-requiring *E.coli* strain is only weak and insignificant. Therefore, this part of the study was conducted by direct plate incorporation.

The study was carried out with and without a metabolic activation system - a liver preparation from male rats pre-treated with Aroclor 1254, and the co-factors required for mixed function oxidase activity (S9 mix).
The correct genotypes of all bacterial test strains used were checked.

Two independent and identical experiments were performed. All bacterial strains were exposed to serial dilutions of Pullulanase, solvent (sterile deionised water), and positive controls. The final concentrations of the test article achieved were 5.0, 2.5, 1.25, 0.625, 0.313, and 0.156 mg per ml (*S.typhimurium)* or per plate (*E.coli*).

The number of revertants per plate was determined by triplicate plating at each dose on selective agar. The number of viable bacteria in each culture was determined by plate count.

No dose-related and reproducible increases in revertants to prototrophy were obtained with any of the bacterial strains exposed to Pullulanase, batch PPY 6454, either in the presence or absence of S-9 mix.

The sensitivity of the individual bacterial strains and the metabolising potential of the S-9 mix were confirmed in both studies by significant increases in number of revertant colonies induced by diagnostic mutagens under similar conditions.

It was concluded, that Pullulanase, batch PPY 6454, did not induce gene mutations in bacteria in either the absence or presence of S-9 mix, when tested under the conditions employed in these studies.

3.2.2 *In vitro* cytogenetic test – cultured human lymphocytes

Covance Laboratories Limited Report No.665/316. Novo Nordisk Study No.996039. Pullulanase: Induction of Chromosome Aberrations in Cultured Human Peripheral Blood Lymphocyte.

The effects on chromosomal structure of exposure to Pullulanase were investigated in cultured human lymphocytes in accordance with the current guidelines of OECD (Guideline 473, July 1997) and the ICH Tripartite Harmonised Guideline on Genotoxicity: Specific Aspects of Regulatory Tests (1995).

Heparinised whole blood cultures from three male donors were established, and division of the lymphocytes was stimulated by adding phytohaemagglutinin (PHA) to the cultures.

Two independent experiments were performed both in the absence and presence of metabolic activation by a rat liver post-mitochondrial fraction (S-9) from animals induced with Aroclor.

Sets of duplicate cultures were treated with the solvent (sterile purified water), test chemical or positive controls (–S-9: 4-Nitroquinoline 1-oxide, +S-9: Cyclophosphamide). Treatments with Pullulanase covered a broad range of doses, separated by narrow intervals, where the highest dose level used was 5000 µg/mL.

The lymphocyte cultures were exposed to the test substance for three hours and cells were harvested 17 hours later. The second experiment included a continuous exposure for 20 hours in the absence of S-9. The test article dose levels for chromosome analysis were selected by evaluating the effect of Pullulanase on mitotic index.
Chromosome aberrations were analysed at three consecutive dose levels. Cells were arrested in metaphase by colchicine and after centrifugation and hypotonic treatment, metaphase spreads were prepared and stained with Giemsa. A total of 200 cells were scored per dose level (100 from each replicate culture) from Pullulanase treatments and negative controls. Slides were scored blind and aberrations classified according to the scheme described by ISCN (ISCN 1985).

The proportion of cells with structural aberrations in all cultures of the solvent controls (purified water) was within the limits of the historical ranges. The positive controls induced statistically significant increases in the proportion of cells with structural aberrations, thus demonstrating the sensitivity of the test procedure and the metabolic activity of the S-9 mix employed.
The highest concentration of Pullulanase chosen for analysis, 5000 µg/mL, produced an approximate 15% and 8% mitotic inhibition (reduction in mitotic index) in the first experiment and 14% and 1% mitotic inhibition in the second experiment in the absence and presence of S-9, respectively.
Cells treated with Pullulanase, either in the absence and presence of S-9, had similar numbers of aberrations to those observed in concurrent solvent controls. There were no increases in aberration frequency that were significantly higher than those observed in the negative controls or that fell outside the historical negative control ranges.
Normal frequencies of cells with numerical aberrations were seen under all treatment conditions.

It was concluded that Pullulanase, under the conditions of test, did not induce chromosome aberrations in cultured human blood lymphocytes.

4. CONCLUSION

Based on the toxicological data summarised above and the fact that the production strain has reportedly a safe industrial history in use, it is concluded that Pullulanase can be considered generally as safe for food applications.

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5.1 Studies

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Novo Nordisk A/S Report No.: 2000-00977-01. NN Study No.: 998072.
Pullulanase (Batch No. PPY 6454): Test for mutagenic activity with strains of Salmonella typhimurium and Escherichia coli.

Covance Laboratories Limited Report No.665/316. Novo Nordisk Study No.996039
Pullulanase: Induction of Chromosome Aberrations in Cultured Human Peripheral Blood Lymphocyte.

5.2 Guidelines

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Appendix 4

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Safety & Toxicology

Date : 09-05-2006
Proj: DEV 00551
File : 2006-20090-01
Ref.: PScK/PBjP

FINALREPORT

Pullulanase GMM, PPY 24933: Test for Mutagenic Activity with Strains of *Salmonella typhimurium* and *Escherichia coli.*

Study No. 20058057

Author :
P. B. Pedersen

Issued by :
Novozymes A/S
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DK- 2880 Bagsværd
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80.226.079

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GLP - COMPLIANCE STATEMENT

REPORT : Pullulanase, PPY 24933: Test for Mutagenic Activity with Strains of *Salmonella typhimurium* and *Escherichia coli* .

STUDY No.: 20058057

A sample of Pullulanase (Batch Number: PPY 24933) was received from Recovery Pilot Plant, Novozymes A/S.

This study was conducted at the department of Safety & Toxicology, Novozymes A/S in compliance with the following current Good Laboratory Practice Regulations:

OECD, ENV/MC/CHEM(98)17, 1998

The one exception to this was the dosing solutions prepared 14th September 2005 and applied in the 2. Experiment with the strains TA1537 and TA100 without incorporation of S9. The preparation of these was not been documented correctly in the raw data.
Based on the results the dosing solutions were prepared and applied correctly and in accordance with the study plan.

Date : 9 May 2006

Peder Bjarne Pedersen
Study Director

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QUALITY ASSURANCE STATEMENT

Report: Pullulanase GMM, batch PPW 24933
Test for mutagenic activity with strains of Salmonella typhimurium and Escherichia coli.

STUDY NUMBER 20058057

The conduct of this study has been subject to appropriate inspections and the report has been reviewed according to the relevant Standard Operation Procedures of Novozymes A/S Quality Assurance..

Inspection/Audit	Dates of inspection	Dates of Audit Report signed by Study Director	Dates of Audit Report signed by Study Management
Protocol	5 sep 2005	15 sep 2005	15 sep 2005
Dosing	13 sep 2005	15 sep 2005	15 sep 2005
Report	13 mar 2006	25 apr 2006	25 apr 2006

09 May 2006
Date

Christina Vesterby
Quality Assurance

1. GENERAL INFORMATION

STUDY	Pullulanase GMM, PPY 24933: Test for Mutagenic Activity with Strains of *Salmonella typhimurium* and *Escherichia coli*. Study No. 20058057
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DATES OF STUDY	Study initiation date : 2005-09-02 Experimental start date : 2005-09-06 Experimental termination : 2005-11-11

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DATE OF FINAL REPORT

Date: 9 May 2006

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2. SUMMARY

Pullulanase (Batch Number: PPY 24933) was examined for mutagenic activity in the bacterial reverse mutation assay using *Salmonella typhimurium* strain TA1535, TA100, TA1537, TA98 and *Escherichia coli* WP2uvrA.

Crude enzyme preparations, like the present Pullulanase, contain the free amino acid L-histidine, most often in an amount, which exceeds the critical concentration for incorporation in the direct standard assay.
To overcome this problem all *Salmonella* strains were exposed to Pullulanase in liquid culture ("treat and plate assay"). Bacteria were exposed to 6 doses of the test substance in a phosphate buffered nutrient broth for 3 hours with 5 mg (dry matter) per ml as highest concentration. After incubation the test substance was removed by centrifugation prior to plating. Two identical and independent experiments were conducted.

Usually the content of tryptophan in enzyme preparations is low and insignificant. Therefore the part of the study comprising *Escherichia coli* was initially conducted with the strain WP2uvrA using the direct plate incorporation assay. 6 doses of the test substance were applied with 5 mg (dry matter) per plate as the highest dose level followed by successive bi-sections between doses.

The study was conducted with and without the metabolic activation system S9 - a liver preparation from male rats, pre-treated with Aroclor 1254, and the co-factors required for mixed function oxidase activity (S9 mix).

Due to toxicity observed in the 1.Exp. the dose range was adjusted in the Exp.2 applying1250µg per mL as highest dose level. No toxicity was observed in test series with metabolic activation and in the plate incorporation assay with the *E.coli* strain.

In general the positive control substances induced significant responses in the appropriate strains in similar conditions as the test article thereby confirming the sensitivity of the test system under the specific conditions of the test.

No treatments of any of the *Salmonella* strains and the *E.coli* strain with Pullulanase resulted in any increases in revertant numbers that meets these criteria for a positive response.

In test series with TA98 with S9 incorporation weak numerical increases was observed. Two additional experiments were conducted and in the final experiment the top dose was raised to 10 mg per mL. Again slight increases in number of revertant colonies was apparent at the highest two concentration analysed in both experiments. The overall evaluation of these results was that the increases observed were considered of no biological significance since the increases were small and within our historical negative control (normal) range. They were only observed at very high doses and without an obvious dose relation.

It was concluded that the results of the bacterial mutagenicity tests described in this report do not indicate that Pullulanase (Batch No. PPY 24933) has the potential to induce mutations of biologically relevance in bacteria in both the absence and presence of a rat liver metabolic activation system (S-9).

3. INTRODUCTION

Bacterial reverse mutation assays have been recognised and used for more than two decades as a rapid, sensitive and reliable method of evaluating the mutagenic potential of chemicals.
Bacterial systems offer several advantages to other test systems. They can be grown in large numbers in a short time, enabling the detection of very rare mutational events. Further, extensive knowledge of bacterial genetics has allowed the construction of special strains, which are more sensitive than the wild-type strains to a variety of agents.
The reversion of bacteria from growth-dependence of a particular amino acid to be able to growth in the absence of that amino acid is the most widely used marker in reverse-mutation assays. The genetic target is small, specific and selective, and the phenotypic effect of the reverse mutation is easily detected.
A wide range of strains within the species *Salmonella typhimurium* (Ames strains) and *Escherichia coli* have been constructed in order to make the test system more sensitive and selective to different classes of chemical mutagens.
By incorporation of the post-mitochondrial supernatant (S9) from the livers of rats pre-treated with an enzyme inducer Aroclor 1254, the metabolising systems present in mammalian cells are mimicked to facilitate the detection of a wide range of pro-mutagens.

This report describes investigations performed to assess the activity of Pullulanase (Batch Number: PPY 24933) in amino acid dependent strains of *Salmonella typhimurium* and *Escherichia coli* capable of detecting both induced frame-shift (TA1537 and TA98) and base-pair substitution mutations (TA1535, TA100 and WP2uvrA).
Pullulanase is a microbial enzyme preparation derived from submerged pure culture fermentations of a non-pathogenic and non-toxigenic strain. It contains a variety of unspent medium residues, including low concentrations of free amino acids like histidine and tryptophan.

This complexity poses several problems during mutagenicity testing in vitro. In the Ames test it composes a rich growth medium to the test bacteria, resulting in completely different and poorly defined environments of exposed cultures compared to control cultures. The main problem is the content of utilizable histidine and sometimes also tryptophan in the test material, since the principle of the Ames test is the histidine auxotrophy of the *Salmonella* tester strains and tryptophan auxotrophy of the *E.coli* strains. Therefore *Salmonella* strains were exposed to Pullulanase in liquid culture ("treat and plate assay").
The part of the study comprising *Escherichia coli* was conducted with the strain WP2uvrA using the direct plate incorporation assay.

Two identically experiments were conducted to demonstrate the reproducibility of the results.

Due to equivocal results in these experiments 2 additionally experiments was carried out with strain TA98 with metabolic activation.

The study was conducted in accordance with 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 any guidelines.

4. MATERIALS

4.1. Test substance

Pullulanase (Batch Number: PPY 24933) was received from Recovery Pilot Plant on the 14. June 2005, and immediately stored in a freezer. The substance was a brown liquid with a declared content of 11.3 % (w/w) dry matter.

A standard solution of 5% (w/v) dry matter was prepared in deionised water and sterilised by filtration. The sterility was confirmed by plate counting.

This solution was stored at 4°C and used as test substance.

4.2. **Positive control substances**

Chemical	Source	Lot.No.
2-Nitrofluorene (2-NF)	Aldrich-Chemie	S 08447-034
9-Aminoacridine (9-AA)	SIGMA Chemical Company	106F-06682
N-Methyl- N'-Nitrosoguanidine (MNNG)	Aldrich-Chemie	15427 LO
N-Ethyl-N'-Nitro-N-Nitrosoguanidine (ENNG)	Aldrich-Chemie	08228 ES
2-Aminoanthracene (2-AA)	SIGMA Chemical Company	S 11804-492
Benzo(α)pyrene (BαP)	SIGMA Chemical Company	063K3494

All positive control substances were dissolved in dimethyl sulphoxide (spectrophotometric grade) obtained from Merck, Darmstadt, Germany.

4.3. **Liver homogenate - S9**
A commercial preparations of S9 from Aroclor 1254 induced Spraque Dawley rats was obtained from ICN Pharmaceuticals, Inc./Cappel Biochemical Division, 1263 S. Chillicothe Rd. Aurora, Ohio 44202. Specifications of the preparation, the enzymatic properties and metabolic activation from the supplier are archived as raw data.

The tubes with S9 were received frozen in dry ice and were immediately stored in a ÷80°C ultra low freezer at Safety & Toxicology, Novozymes.

4.4. **Plates**
As selective substrate for reverted bacteria Vogel-Bonner medium E agar plates with 2% glucose were prepared in-house as described in Appendix 1.

All plates were stored at 4°C in closed plastic bags and examined for contamination and dryness before use.

4.5. **Bacteria**
Salmonella typhimurium

Four strains of *Salmonella typhimurium* were used :
S. typhimurium TA1535
S. typhimurium TA100
S. typhimurium TA1537
S. typhimurium TA98

All these strains contain mutations in the histidine operon, thereby imposing a requirement for histidine in the growth medium. They all contain GC base-pairs at the site of the histidine mutation, and are therefore selective for agents which react predominantly with these bases. Three mutations in the histidine operon are involved:

his G 46 (TA1535 and TA100) is a missense mutation which is reverted to prototrophy by a variety of mutagens that cause base-pair substitutions.
his C 3076 (TA1537) contains a frame-shift which appears to have added a GC base-pair. This mutation is reverted for example by 9-Aminoacridine and epoxides of polycyclic hydrocarbon.
his D 3052 (TA98) also contains a frame-shift mutation with a sequence of repeated GC, which is reverted with the deletion of 2 of these base-pairs. It is readily reverted by aromatic amines and derivatives.

All 4 strains contain the deep rough (rfa) mutation, which deletes the polysaccharide side chain of the polysaccharide coat of the bacterial cell surface. This deletion increases cell permeability to more hydrophobic substances and, furthermore, greatly decreases the pathogenicity of these organisms.
The uvrB deletion renders the strains incapable of excision repair, making them more sensitive both to the mutagenic and lethal effects of a wide variety of mutagens (e.g. polyaromatic hydrocarbons), since the strains can not excise DNA adducts.
These 2 deletions include the nitrate reductase (chl) and biotin (bio) genes also.

Strain TA98 and TA100 are derived from strain TA1538 and TA1535 respectively by the addition of a plasmid, pKM101, which confers resistance to ampicillin. This plasmid also carries a gene (muc^+), which in some strains ($recA^+/lexA^+$) have proven to participate in "SOS" DNA-repair. This repair pathway is induced by DNA damage and confers resistance to the lethal effects of many mutagens at the expense of increased mutability. Bacteria carrying pKM101 have therefore a higher spontaneous mutation rate.

Escherichia coli

One strain was used: *Escherichia coli* WP2uvrA

This strain contains an ochre mutation in the trpE locus and can be mutated to tryptophan independence either by a base-pair reversion of an A-T base-pair in the trpE locus, or more likely, by a base-pair substitution within a number of transfer RNA loci elsewhere in the chromosome. The latter causes the original defect to be suppressed (ochre suppression) and involves only base-pair substitution transitions at G-C base-pairs.
Like the uvrB mutation in the Salmonella strains, the uvrA mutation causes the bacteria to be deficient in the excision of bulky lesions from the DNA, so, it is more readily mutated by certain agents (ultraviolet radiation, polycyclic hydrocarbons).

4.6. **Bacterial cultures**
The test strains of *Salmonella typhimurium* LT2 were obtained from Prof. B.N. Ames, Biochemistry Department, University of California, Berkeley, CA 94720, U.S.A.
Escherichia coli WP2uvrA was obtained from The National Collections of Industrial and Marine Bacteria Ltd., Aberdeen, Scotland.
New batches of culture stocks frozen in 8% dimethyl sulphoxide are prepared at intervals from a central stock held in liquid nitrogen. They are regularly checked for appropriate amino acid requirement, spontaneous reversion rate, genetic characters and response to diagnostic mutagens.
Samples of each strain were grown up overnight in Nutrient broth in a 37 ± 1°C water bath with shaking. Fresh cultures were prepared before each test.

4.7. **S9 mix**
Composition of a 10% V/V S9 mix (final concentrations):
Co-factors:
-phosphate buffer (0.2M, pH 7.4) ..... 100 mM
-salts (1.65M KCl, 0.4 M MgCl) ..... 33 and 8 mM
-glucose-6-phosphate, mono-Na salt (0.2M) ..... 5 mM
-NADP, di-Na salt (0.1M) ..... 4 mM
S9 preparation ..... 10% V/V

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A freshly prepared solution of the co-factors was filter-sterilised by passage through a 0.2 μm membrane filter and mixed 9:1 (v/v) with freshly thawed still cold S9 preparation.
This S9 mix was prepared freshly each day, and immediately used. Unused reagent was discarded.

4.8. **Test material**
Serial dilutions of the sterile standard solution (4.1.) were prepared in sterile deionised water corresponding to the final dose levels:

Salmonella strains; 1.Exp. with and without S9 and 2.Exp. with S9:
5000 - 2500 - 1250 - 625 - 313 and 156 μg test substance per ml.

Salmonella strains; 2.Exp. without S9:
1250 - 625 - 313 - 156 – 78 and 39 μg test substance per ml.

E.coli strain; 1.Exp. and 2.Exp. with and without S9:
5000 - 2500 - 1250 - 625 - 313 and 156 μg test substance per plate.

The dilutions were prepared freshly each day just before use.

4.9. **Top agar**
0.6% soft agar was sterilised by autoclaving.
Bottles with 100 ml melted soft agar were kept at about 55°C and added 10 ml L-histidine/biotin solution for strains of *Salmonella* or 10 ml tryptophan solution for *Escherichia coli*. This molten agar was divided into 2 ml aliquots in sterile glass tubes and placed in a "Driblock" (Techne) at 45 ± 1°C.

5. METHODS

5.1. Treat and plate assay
This procedure was applied in test series with all strains of *Salmonella typhimurium*.

For each assay sterile tubes were added:
- 4 ml Nutrient broth
- 4 ml S9 mix or 0.2M phosphate buffer (pH 7.4)
- 1 ml bacterial culture
- 1 ml test substance solution (6 doses) or diagnostic mutagen solution (positive control) or sterile deionised water (solvent control).

These incubation mixtures were incubated with shaking at 37 ± 1°C for 3 hours.
After incubation all bacterial suspensions were washed 2 times by centrifugation for 10 minutes at 2500 rpm. After the first washing the bacterial pellets were resuspended in 5 ml phosphate buffer (pH 7.4, 0.2M) and finally they were re-suspended in 1 ml phosphate buffer.
Tubes with top agar were added 0.1 ml of all washed bacterial suspensions.

5.2. Plate incorporation assay
This procedure was applied in the test series with *Escherichia coli* WP2uvrA.

Tubes with molten top agar were added:
100 μl of the dosing solutions of the test article or solvent (negative control) or standard mutagen (positive control).
100 μl bacterial culture.
500 μl S9 mix in test series with metabolic activation or 500 μl 0,2 M phosphate buffer in series without metabolic activation.

5.3. Selective incubation

For each dose of the test substance and the standard mutagens 3 similar tubes with top agar were prepared and 5 tubes were prepared for the solvent control.
These tubes were poured on to minimal glucose agar plates. When the soft agar set, the plates were inverted and incubated at 37 ± 1°C for about 48-72 hours. After incubation the numbers of revertant colonies were counted automatically ("Cardinal" - Perceptive Instruments). Plates with less than about 20 colonies were counted manually.

5.4. Viable cell count

0.1 ml aliquots of a 10^{-6} dilution of each bacterial suspension were poured on to minimal glucose agar plates (added the required amino acids in excess) in duplicates.

5.5. Controls

The following controls were run with each experiment :
Genotype checking:
Sensitivity for crystal violet (rfa-character)- (except E. coli)
Sensitivity for Mitomycin C (uvrB).
Resistance to ampicillin (pKM101).
0.1 ml bacterial culture was spread on to complete agar medium. To the surface of the dried plate was added a disc of ampicillin/(Rosco Neo-Sensitabs) and two 6 mm ϕ sterile filter discs, one with 10µl 0.1% crystal violet and the other with 10µl 0.01% Mitomycin C. The plate was incubated for 48-72 hours at 37 ± 1°C.

Sterility of S9 mix:
0.1 ml S9 mix was plated on to complete medium and incubated for 48-72 hours at 37±1°C.

Diagnostic mutagens were used for each strain with and without S9 mix, as follows:

Mutagen	S9	Strain	µg/plate	µg/ml
MNNG	-	TA 1535		1,0
MNNG	-	TA 100		1,0
2-NF	-	TA 98		20,0
9-AA	-	TA 1537		2,0
ENNG	-	WP2uvrA	2,0	
2-AA	+	TA 98		5,0
2-AA	+	TA 1537		5,0
2-AA	+	TA 1535		5,0
2-AA	+	TA 100 (1.Exp.)		5,0
B(a)P	+	TA 100 (2.Exp.)		10,0
2-AA	+	WP2uvrA	20,0	

Deviation form protocol:
The protocol states that Benzo(a)pyrene (B(a)P) is used as positive control substance with the strain TA100 when S9 is incorporated. However in 1.Exp. 2-Aminoanthracene (2-AA) was applied by a mistake. 2-AA is a fully acceptable alternative to B(a)P in this strain.

6. RESULTS AND DISCUSSION

6.1 Genetic characters

All strains used in these experiments were sensitive to Mitomycin C. All *Salmonella* strains were sensitive to crystal violet. Further the *Salmonella* strains TA98 and TA100 were resistant to ampicillin. These results are as expected.

6.2 Negative control levels

All negative control values presented in this report are within the normal ranges experienced in our laboratory with these strains of *Salmonella typhimurium* and *Escherichia coli*. The values with TA98 and TA100 are in the low end of our historical data. However these cultures responded satisfactory to the respective positive control substances. In general the spontaneous revertant levels are lower in a treat and plate assay than in the standard plate incorporation assay.

6.3 Diagnostic mutagens

The increases in revertant colony numbers induced by positive control chemicals were all within the ranges experienced in our laboratory under the specific conditions applied in the present investigation. The one exception to this was in the first experiment with the *Salmonella* strain TA1537 without S9 where 9-Aminoacridine induced a significant positive response but relatively low compared to our historical data. It is our experience that these responses to diagnostic mutagens are rather variable in the treat and plate assay.

6.4 Pullulanase (Batch Number: PPY 24933)

The results are represented in Table 1-11.

Pullulanase is a fluid enzyme preparation. It contains an abundance of various nutrients, and composes a growth medium to the test bacteria. This means, that comparison of viable counts between exposed cultures and control culture in a "treat and plate" assay reflects growth stimulation/inhibition as well as cell killing.
In Exp.1 toxicity was observed when the *Salmonella* strains TA1537, TA100 and TA1535 were treated with doses higher than approximately 1 mg per mL in the absence of metabolic activation. Therefore the dose range was adjusted in the Exp.2 applying1250µg per mL as highest dose level. No toxicity was observed in test series with metabolic activation and in the plate incorporation assay with the *E.coli* strain.

It is our experience, that in a liquid culture assay, where bacteria are exposed to different doses of the test substance in separate liquid cultures for a certain time, the revertant levels randomly fluctuate more than in the direct "plate incorporation assay." Some of the results with the *Salmonella* strains reflect this circumstance.

We consider a test substance as positive when it has induced at least a doubling in the mean number of revertants per plate compared to the appropriate solvent control in one or more of the strains, in the presence or absence of S9, if this response is dose related (at least 3 doses) and reproducible.

No treatments of any of the *Salmonella* strains and the *E.coli* strain with Pullulanase resulted in any increases in revertant numbers that meets these criteria for a positive response.

However in the test series with TA98 with S9 incorporation (Table 1 and 6) weak numerical increases was observed which in the 2.Exp just exceeded a doubling at the highest dose level. In both experiments these increases were set against a rather low concurrent solvent control, and all colony counts fell within our historical negative control range for this strain (App. 1). Due to this equivocal result these test series was repeated twice (Exp.3 and 4, Table 11). In the final experiment the top dose was raised to 10 mg per mL which correspond to twice the acceptable top dose for this type of study according to current regulatory guidelines. Slight increases in number of revertant colonies was apparent at the highest two concentration analysed in both experiments, but without dose relation. In the 3.Exp. the increases were again observed against a low solvent control value. In the 4.Exp. the increases were less than 50% compared to the solvent control.

The overall evaluation of these results is therefore that the increases observed are considered of no biological significance since the increases are marginal and within our historical negative control (normal) range. They are only observed at very high doses and without an obvious dose relation.

7. CONCLUSION

It is concluded that the results of the bacterial mutagenicity tests described in this report do not indicate that Pullulanase (Batch No. PPY 24933) has the potential to induce mutations of biologically relevance in bacteria in both the absence and presence of a rat liver metabolic activation system (S-9).

Table 1. Number of revertant colonies per plate obtained with *Salmonella typhimurium* LT2 strain **TA98** following exposure to Pullulanase, (Batch No. PPY 24933), in the presence and absence of metabolic activation in a liquid culture assay.

1. Experiment

µg per ml incubation mixture	Strain +/÷ S9	Revertants		Viable cells [1]		Strain +/÷ S9	Revertants		Viable cells [1]	
		Single plates	Mean	Single plates	Mean		Single plates	Mean	Single plates	Mean
5000	TA 98 ÷ S9	8 12 21	14	47 47	47	TA 98 + S9	20 14 21	18	161 161	161
2500		11 5 16	11	55 40	48		20 26 30	25	194 207	201
1250		17 10 15	14	81 67	74		17 18 20	18	226 195	211
625		15 13 24	17	153 162	158		15 18 21	18	246 248	247
313		25 15 17	19	85 140	113		14 13 17	15	260 274	267
156		21 13 18	17	185 163	174		21 21 27	23	270 278	274
Sterile water		22 16 22 14 13	17	213 182	198		12 16 10 17 15	14	152 150	151
2-Nitro-fluorene 20 µg		895 910 926	910	115 136	126					
2-Amino-anthracene 5 µg							715 731 735	727	144 117	131

1) Corresponding to no. of viable cells x10^6 on revertant plates.

Table 2. Number of revertant colonies per plate obtained with *Salmonella typhimurium* LT2 strain **TA1537** following exposure to Pullulanase, (Batch No. PPY 24933), in the presence and absence of metabolic activation in a liquid culture assay.

1. Experiment

µg per ml incubation mixture	Strain +/÷ S9	Revertants		Viable cells [1]		Strain +/÷ S9	Revertants		Viable cells [1]	
		Single plates	Mean	Single plates	Mean		Single plates	Mean	Single plates	Mean
5000	TA 1537 ÷ S9	4 4 7	5	2 5	4	TA 1537 + S9	13 16 15	15	123 139	131
2500		4 4 5	4	2 4	3		20 9 15	15	121 147	134
1250		4 5 1	3	10 9	10		18 15 18	17	137 142	140
625		11 9 13	11	152 166	159		9 11 21	14	162 125	144
313		15 23 20	19	175 136	156		17 19 22	19	125 139	132
156		16 12 11	13	132 151	142		21 22 23	22	158 139	149
Sterile water		16 21 19 22 11	18	175 184	180		18 16 14 11 21	16	81 86	84
9-Amino-acridine 2 µg		524 563 563	550	147 125	136					
2-Amino-anthracene 5 µg							142 140 133	138	41 68	55

1) Corresponding to no. of viable cells x10^6 on revertant plates.

Table 3. Number of revertant colonies per plate obtained with *Salmonella typhimurium* LT2 strain **TA100** following exposure to Pullulanase, (Batch No. PPY 24933) in the presence and absence of metabolic activation in a liquid culture assay.

1. Experiment

µg per ml incubation mixture	Strain +/÷ S9	Revertants		Viable cells [1]		Strain +/÷ S9	Revertants		Viable cells [1]	
		Single plates	Mean	Single plates	Mean		Single plates	Mean	Single plates	Mean
5000	TA 100 ÷ S9	41 41 44	42	4 3	4	TA 100 + S9	92 97 101	97	No determination due to error	
2500		39 49 37	42	11 5	8		110 121 110	114		
1250		55 39 45	46	18 15	17		101 117 103	107		
625		75 50 68	64	145 169	157		52 86 72	70		
313		65 58 74	66	101 114	108		66 70 87	74		
156		79 50 72	67	137 97	117		63 59 82	68		
Sterile water		76 99 63 68 73	76	104 124	114		82 86 104 97 58	85		
MNNG 1 µg		2843 2932 3194	2990	119 114	117					
2-Amino-anthracene 5µg							1794 1652 1911	1786		

1) Corresponding to no. of viable cells x10^6 on revertant plates.
2) Abbreviation: MNNG = N-Methyl-N'-Nitro-Nitrosoguanidine.

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Table 4. Number of revertant colonies per plate obtained with *Salmonella typhimurium* LT2 strain **TA1535** following exposure to Pullulanase, (Batch No. PPY 24933) in the presence and absence of metabolic activation in a liquid culture assay.

1. Experiment

µg per ml incubation mixture	Strain +/÷ S9	Revertants		Viable cells [1]		Strain +/÷ S9	Revertants		Viable cells [1]	
		Single plates	Mean	Single plates	Mean		Single plates	Mean	Single plates	Mean
5000	TA 1535 ÷ S9	5 2 7	5	3 6	5	TA 1535 + S9	10 6 5	7	156 341	249
2500		3 6 2	4	6 6	6		11 8 6	8	185 179	182
1250		2 3 5	3	34 49	42		6 7 11	8	227 269	248
625		5 5 8	6	129 147	138		7 2 5	5	202 207	205
313		1 1 3	2	92 69	81		2 8 9	6	302 294	298
156		6 3 4	4	146 121	134		6 8 6	7	276 313	295
Sterile water		7 1 3 8 6	5	117 135	126		5 4 6 9 7	6	193 226	210
MNNG 1 µg		4537 4336 4223	4365	124 163	144					
2-Amino-anthracene 5µg							155 129 172	152	77 cont.	77

1) Corresponding to no. of viable cells x10^6 on revertant plates.
2) Abbreviation: MNNG = N-Methyl-N'-Nitro-Nitrosoguanidine.

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Table 5. Number of revertant colonies per plate obtained with *Escherichia coli* WP2uvrA in the direct plate incorporation assay following exposure Pullulanase, PPY 24933.

1. Experiment

Test substance-concentration (µg/plate)	Number of revertants (number of colonies/plate)			
	Without S9		With S9	
	Singleplates	Mean	Singleplates	Mean
Solvent control	38 45 32 40 49	41	48 38 52 34 49	44
5000	32 21 41	31	30 38 41	36
2500	47 54 44	48	27 41 31	33
1250	43 47 44	45	48 33 32	38
625	33 44 48	42	42 26 50	39
313	38 31 42	37	47 42 38	42
156	43 47 36	42	36 49 34	40
Positive controls	ENNG 2 µg/plate		2-Amino-anthracene 20 µg/plate	
Number of colonies/plate	422 310 358	363	512 483 462	486
Viable count of bacterial cultures x 10^7	304 319	312	304 319	312

Abbreviation: ENNG = 1-Ethyl-3-Nitro-1-Nitrosoguanidine.

Table 6. Number of revertant colonies per plate obtained with *Salmonella typhimurium* LT2 strain **TA98** following exposure to Pullulanase, (Batch No. PPY 24933) in the presence and absence of metabolic activation in a liquid culture assay.

2. Experiment

µg per ml incubation mixture	Strain +/÷ S9	Revertants		Viable cells [1]		Strain +/÷ S9	Revertants		Viable cells [1]	
		Single plates	Mean	Single plates	Mean		Single plates	Mean	Single plates	Mean
5000	TA 98 ÷ S9	13 12 12	12	77 71	74	TA 98 + S9	36 40 32	36	163 142	153
2500		34 20 34	29	107 112	110		27 29 23	26	222 242	232
1250		34 26 22	27	132 99	116		31 27 23	27	280 274	277
625		29 27 27	28	191 174	183		27 20 25	24	238 234	236
313		25 43 29	32	146 123	135		20 20 22	21	186 190	188
156		27 20 38	28	168 174	171		15 20 20	18	229 215	222
Sterile water		18 19 24 36 17	23	158 136	147		13 9 16 21 13	14	196 179	188
2-Nitro-fluorene 20 µg		884 849 805	846	138 130	134					
2-Amino-anthracene 5 µg							2207 2434 2144	2262	125 129	127

1) Corresponding to no. of viable cells x10^6 on revertant plates.

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Table 7. Number of revertant colonies per plate obtained with *Salmonella typhimurium* LT2 strain **TA1537** following exposure to Pullulanase, (Batch No. PPY 24933) in the presence and absence of metabolic activation in a liquid culture assay.

2. Experiment

µg per ml incubation mixture	Strain +/÷ S9	Revertants		Viable cells [1]		Strain +/÷ S9	Revertants		Viable cells [1]	
		Single plates	Mean	Single plates	Mean		Single plates	Mean	Single plates	Mean
5000	TA 1537 ÷ S9					TA 1537 + S9	11 7 10	9	146 177	162
2500							15 14 13	14	172 119	146
1250		6 5 1	4	33 44	39		6 15 8	10	287 279	283
625		15 19 10	15	157 158	158		8 6 5	6	162 156	159
313		11 13 10	11	171 144	158		6 7 9	7	285 312	299
156		15 10 11	12	120 172	146		13 5 14	11	201 228	215
78		12 27 16	18	112 106	109					
39		9 10 5	8	119 109	114					
Sterile water		21 20 15 13 14	17	101 108	105		3 9 8 12 9	8	183 178	181
9-Amino-acridine 2 µg		222 281 236	246	109 124	117					
2-Amino-anthracene 5 µg							136 162 144	147	115 81	98

1) Corresponding to no. of viable cells x10^6 on revertant plates.

Table 8. Number of revertant colonies per plate obtained with *Salmonella typhimurium* LT2 strain **TA100** following exposure to Pullulanase, (Batch No. PPY 24933) in the presence and absence of metabolic activation in a liquid culture assay.

2. Experiment

µg per ml incubation mixture	Strain +/÷ S9	Revertants		Viable cells [1]		Strain +/÷ S9	Revertants		Viable cells [1]	
		Single plates	Mean	Single plates	Mean		Single plates	Mean	Single plates	Mean
5000	TA 100 ÷ S9					TA 100 + S9	85 72 58	72	69 108	89
2500							77 90 86	84	353 293	323
1250		53 32 43	43	33 34	34		80 83 88	84	217 173	195
625		70 55 68	64	112 121	117		91 86 75	84	184 201	193
313		49 58 80	62	130 135	133		77 72 82	77	298 280	289
156		52 50 75	59	96 103	100		86 68 63	72	318 359	339
78		38 59 56	51	96 86	91					
39		61 77 42	60	135 133	134					
Sterile water		69 66 55 64 59	63	105 88	97		69 66 54 74 69	66	185 182	184
MNNG 1 µg		1931 1951 1812	1898	80 92	86					
Benzo(a)-pyrene 10µg							513 466 465	481	132 172	152

1) Corresponding to no. of viable cells x10^6 on revertant plates.
2) Abbreviation: MNNG = N-Methyl-N'-Nitro-Nitrosoguanidine.

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Table 9. Number of revertant colonies per plate obtained with *Salmonella typhimurium* LT2 strain **TA1535** following exposure to Pullulanase, (Batch No. PPY 24933) in the presence and absence of metabolic activation in a liquid culture assay.

2. Experiment

µg per ml incubation mixture	Strain +/÷ S9	Revertants		Viable cells [1]		Strain +/÷ S9	Revertants		Viable cells [1]	
		Single plates	Mean	Single plates	Mean		Single plates	Mean	Single plates	Mean
5000	TA 1535 ÷ S9					TA 1535 + S9	4 3 2	3	199 132	166
2500							5 2 5	4	261 233	247
1250		2 2 3	2	72 54	63		8 5 4	6	343 312	328
625		3 3 3	3	162 135	149		5 2 5	4	309 386	348
313		2 6 6	5	217 223	220		4 12 6	7	388 331	360
156		4 2 6	4	206 207	207		3 1 6	3	363 467	415
78		3 6 4	4	163 147	155					
39		5 5 5	5	168 147	158					
Sterile water		4 3 4 6 3	4	139 146	143		2 4 3 5 3	3	301 296	299
MNNG 1 µg		3082 2907 2761	2917	142 155	149					
2-Amino-anthracene 5 µg							126 129 142	132	196 166	181

1) Corresponding to no. of viable cells x10^6 on revertant plates.
2) Abbreviation: MNNG = N-Methyl-N'-Nitro-Nitrosoguanidine.

Table 10. Number of revertant colonies per plate obtained with *Escherichia coli* WP2uvrA in the direct plate incorporation assay following exposure to Pullulanase, PPB 24933.

2. Experiment

Test substance-concentration (µg/plate)	Number of revertants (number of colonies/plate)			
	Without S9		With S9	
	Singleplates	Mean	Singleplates	Mean
Solvent control	42 41 36 45 31	39	32 26 36 25 25	29
5000	31 44 33	36	50 34 33	39
2500	42 52 34	43	31 31 32	31
1250	22 28 38	29	49 36 32	39
625	34 39 45	39	27 37 33	32
313	24 36 32	31	26 39 29	31
156	41 43 33	39	34 35 29	33
Positive controls	ENNG 2 µg/plate		2-Amino-anthracene 20 µg/plate	
Number of colonies/plate	200 242 250	231	587 435 439	487
Viable count of bacterial cultures x 10^7	274 324	299	274 324	299

Abbreviation: ENNG = 1-Ethyl-3-Nitro-1-Nitrosoguanidine.

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Table 11. Number of revertant colonies per plate obtained with *Salmonella typhimurium* LT2 strain **TA98** following exposure to Pullulanase, (Batch No. PPY 24933) in the presence and absence of metabolic activation in a liquid culture assay.

	3. Experiment				4. Experiment			
µg per ml incubation mixture	Revertants		Viable cells [1]		Revertants		Viable cells [1]	
	Single plates	Mean	Single plates	Mean	Single plates	Mean	Single plates	Mean
10000					31 28 30	30	215 173	194
5000	39 21 33	31	28 4	16	37 21 39	32	309 262	286
2500	28 27 41	32	75 61	68	31 18 18	22	342 329	336
1250	17 12 22	17	100 88	94	28 23 26	26	304 294	299
625	21 17 18	19	161 196	179	23 20 29	24	415 359	387
313	18 12 17	16	60 8	34	12 21 25	19	271 272	272
156	12 9 26	16	93 86	90				
Sterile water	8 11 16 16 16	13	88 61	75	25 16 18 23 13	19	275 266	271
2-Amino-anthracene 5 µg	2638 2773 2635	2682	61 118	90	2337 2253 2362	2317	229 231	230

1) Corresponding to no. of viable cells x10^6 on revertant plates.

APPENDIX 1

Historical control data

Negative control (water) ranges for *S. typhimurium* strains obtained in the treat and plate assay (SOP: TOX-SM-0809) and an *E. coli* strain obtained in the plate incorporation assay (SOP: TOX-SM-0806).

Strain	S9	Number of determinations *)	Mean No. of revertants	SD	Range **) lower	upper
TA1535	÷	16	6.5	± 1,7	4	9
	+	14	6.4	± 1,9	3	9
TA100	÷	19	73.1	± 7,0	58	94
	+	21	81,3	± 15,1	55	110
TA1537	÷	16	10.9	± 5,1	4	22
	+	19	11.1	± 5,7	4	25
TA98	÷	18	17.7	± 5,4	10	28
	+	23	22.9	± 7,4	13	41
WP2 uvrA	÷	22	40.9	± 10,3	24	60
	+	23	44.7	± 15,1	20	84

*) The above are pooled data from a number of independent determinations selected from studies conducted within 2005. Only determinations, which were obviously vitiated by errors, have been omitted.

**) Ranges stated are the maximum and minimum mean spontaneous revertant counts from the data sets sampled.

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APPENDIX 2

Historical control data

Positive control ranges for *S. typhimurium* strains obtained in the treat and plate assay and an *E. coli* strain obtained in the plate incorporation assay.

Strain	S9	Number of determinations *)	Chemical	Mean No. of revertants	SD	Range **) lower	upper
TA1535	÷	15	MNNG 1 µg/mL	3776	1808	699	5783
	+	14	2-AA 5 µg/mL	126	37	53	197
TA100	÷	16	MNNG 1 µg/mL	2970	1611	493	4846
	+	8	2-AA 5 µg/mL	1159	693	205	2312
	+	16	BaP 10 µg/mL	348	78	160	481
TA1537	÷	16	9-AA 2 µg/mL	1039	839	166	3521
	+	19	2-AA 5 µg/mL	148	48	50	228
TA98	÷	18	2-NF 20 µg/mL	888	144	609	1264
	+	21	2-AA 5 µg/mL	1825	645	203	2682
WP2 uvrA	÷	19	ENNG 2 µg/plate	722	428	200	1635
	+	20	2-AA 20 µg/plate	546	233	228	1051

Abbreviations:

2NF: 2-nitrofluorene
MNNG: N-methyl-N'-nitro-N-nitrosoguanidine
ENNG: N-Ethyl-N'-nitro-N-nitrosoguanidine
2-AA: 2-aminoanthracene
BaP: Benzo(a)pyrene
9-AA: 2-aminoacridine

*) The above are pooled data from a number of independent determinations from studies conducted within 2005. Only determinations, which were obviously vitiated by errors, have been omitted.

**) Ranges stated are the maximum and minimum mean induced revertant colony counts from the data sets sampled.

APPENDIX 3

PREPARATION OF MEDIA

1. **Top-agar - histidine-deficient soft agar**

Agar, Merck	0.6 g
NaCl	0.6 g
Distilled water to	100 ml

The medium was autoclaved for 15 minutes at 121°C. After cooling to about 60°C, 10 ml of a sterile aqueous solution of 0.5 mM biotin - 0.5 mM histidine was added aseptically.

2. **Nutrient broth - histidine-rich broth**

Difco nutrient broth	8 g
NaCl	5 g
Distilled water to	1 litre

The medium was autoclaved for 15 minutes at 121°C.

3. **Nutrient agar - histidine-rich agar medium**

Agar, Merck	15 g
Oxoid nutrient broth No. 2	25 g
Distilled water to	1 litre

The medium was autoclaved for 15 minutes at 121°C.

4. **Minimal medium**

This was Vogel-Bonner minimal "E" medium with 2% glucose, prepared as follows :

Solution A (Vogel-Bonner medium E, 20X)

$MgSO_4$ $7H_2O$	4 g
Citric acid, monohydrate	40 g
K_2HPO_4	200 g
$NaHNH_4$ $4H_2O$	70 g
Distilled water to	1000 ml

The solution was sterilized by filtration.

Solution B (40% glucose)

Glucose	40 g
Distilled water to	100 ml

This solution was sterilized by filtration.

Solution C (Agar base)

Agar, Merck	16.7 g
Distilled water to	1000 ml

Solution C was autoclaved for 15 minutes at 121°C. After cooling to 60°C, 450 ml of solution C was aseptically added 25 ml solution A and 25 ml solution B.

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Safety & Toxicology

Date: 24. May 2006
File: 2006-20714-01
Ref.: JLic/PScK
Proj.: Dev 00551

FINALREPORT

Pullulanase, batch PPY 25645
In Vitro Cytotoxicity Test:
Neutral Red Uptake in L929 Monolayer Culture

Study No.: 20068046

Author:
Jens Lichtenberg

Issued by:
Novozymes A/S
Krogshøjvej 36
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GLP – COMPLIANCE STATEMENT

REPORT : Pullulanase, batch PPY 25645.
In Vitro Cytotoxicity Test: Neutral Red Uptake in L929 Monolayer Culture.

STUDY No.: 20068046

The sample of Pullulanase, batch PPY 25645 was received from Recovery Pilot Plant, Novozymes A/S.

This study was conducted at the department of Safety & Toxicology, Novozymes A/S in compliance with the following current Good Laboratory practice Regulations:

OECD Principles of GLP, ENV/MC/CHEM (98) 17, 1998.

Date: 2 June 2006

Jens Lichtenberg
Study Director

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QUALITY ASSURANCE STATEMENT

Report. Pullulanase, batch PPY 25645
In Vitro Cytotoxicity Test: Neutral Read Uptake in L929 Monolayer Culture

STUDY NUMBER 20068046

The conduct of this study has been subject to appropriate inspections and the report has been reviewed according to the relevant Standard Operation Procedures of Novozymes A/S Quality Assurance..

Inspection/Audit	Dates of inspection	Dates of Audit Report signed by Study Director	Dates of Audit Report signed by Study Management
Protocol	23 mar 2006	27 mar 2006	3 apr 2006
Colouring and extraction	29 mar 2006	7 apr 2006	7 apr 2006
Report	5 maj 2006	22 maj 2006	24 maj 2006

2 june 2006
Date

Sonia Nazneen Skotner
Quality Assurance

1. GENERAL INFORMATION

STUDY	Pullulanase, batch PPY 25645 *In Vitro* Cytotoxicity Test: Neutral Red Uptake in L929 Monolayer Culture. Study No. 20068046
STUDY DIRECTOR	Jens Lichtenberg, DVM, DABT Safety & Toxicology Novozymes A/S Krogshøjvej 36 DK – 2880 Bagsværd
MANAGEMENT/ SPONSOR	Merete Simonsen Safety & Toxicology Novozymes A/S Krogshøjvej 36 DK - 2880 Bagsværd
TEST FACILITIES	Safety & Toxicology Novozymes A/S Krogshøjvej 36 DK - 2880 Bagsværd
ARCHIVE	QM Central Archive Novozymes A/S Krogshøjvej 36 DK - 2880 Bagsværd
DATES OF STUDY	Study Initiation Date: 17 March 2006 Experimental Starting Date: 27 March 2006 Experimental Completion Date: 07 April 2006

PERSONNEL INVOLVED IN THE STUDY

TECHNICIANS Pia Schock Kristensen – Safety & Toxicology

DATE OF FINAL REPORT

Date: 2 June 2006

Jens Lichtenberg
Safety & Toxicology

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2. SUMMARY

Pullulanase, batch PPY 25645, was examined for cytotoxic potential in an *in vitro* bioassay, the Neutral Red Uptake assay, using L929 mouse fibroblast cells grown as monolayer culture in 96-well microplates.

The time of exposure for the test substance and positive control was 24 hours. The concentration of the test substance required to reduce the viability of the treated test system to 50% of that of the untreated control test system was determined as the endpoint (NRU_{50}).
The NRU_{50} value for Pullulanase, batch PPY 25645, was estimated to be approximately 18 mg/mL.
This result indicates that Pullulanase, batch PPY 25645, is non-cytotoxic, in the present *in vitro* Neutral Red Uptake assay applying the mouse fibroblast cell line L929 as test system.

3. INTRODUCTION

The neutral red uptake (NRU) assay is a quantitative, colorimetric method to measure the cell viability. The assay procedure is a cell survival/viability assay based on the ability of viable cells to incorporate and bind Neutral Red (NR), a supravital dye. NR is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion and accumulates intracellularly in lysosomes. Alteration of the cell surface or the sensitive lysosome membrane leads to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics (test substance) result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable and damaged or dead cells, and this is the basis of the assay.

The test system L929 is an established mouse fibroblast cell line. It was selected for the ease with which these cells are maintained and grown as monolayer culture and it is commonly used as first order test system for general cytotoxicity.

The L929 cells, when appropriately maintained in culture in a sub confluent state, continuously divide and multiply over time. A toxic chemical, regardless of site or mechanism of action, will interfere with this process and result in cell death and/or a reduction of the growth rate as reflected by cell number. The amount of neutral red uptake by the cells after exposure to the test substance is an indication of the toxicity of the test article.

Cytotoxicity of test substance is expressed as the concentration of test material required to reduce the uptake of NR to 50% of untreated control cells after 24 hours of exposure.

4. MATERIALS

4.1. Media and reagents

- The growth medium Earle's Minimum Essential (EMEM) was supplemented with Fetal Bovine Serum (FBS), $NaHCO_3$ and antibiotics/ antimycotics. The medium

was stored at 4°C for not more than a month. Prior to use the medium was allowed to obtain room temperature.

- 0.05% Trypsin/EDTA solution was thawed and stored at 4°C for not more than a month. The solution was allowed to obtain room temperature prior to use.

- Neutral Red (NR) stock solution was made by dissolving 5 mg NR dye pr. mL Milli-Q water. The solution was sterilized by filtration and stored at room temperature for max. 6 months.

- NR Medium was made by dissolving 500 µL NR stock solution in 50 mL EMEM medium.
  The NR medium was incubated overnight at 37°C and centrifuged at 1100 x *g* for 5 min before use. The solution was sterilized by filtration before use.

- NR Desorb solution was prepared from glacial acetic acid 1 % v/v, ethanol 50 % v/v and Milli-Q water 49 % v/v. The NR desorb solution was stored in a tightly closed bottle at room temperature for no longer than 3 months.

4.2 Test substance

The test substance, Pullulanase, batch PPY 25645, a brown liquid at room temperature, was received from Recovery Pilot Plant on 13 March 2006 and immediately stored in a freezer.

Enzyme activity:	5710 NPUN/g
Specific gravity:	1.048 g/mL
TOS (Total Organic Solids):	9.6 % w/w

A stock solution of 30 mg/mL was prepared in growth medium the day before being applied to the test system. The stock solution was stored at 4°C until dilution and use. Stability data from NZ Study No. 20068021 (Characterization-report of tox-batch, Pullulanase, batch PPY 25645, Luna No. 2006-04793-01/KM) demonstrates that the test material is stable for at least 24 hours at 4 °C. Osmolality and pH of the stock solution was measured to be 322 mOsm/kg and pH 7.5, respectively. The osmolality was found to be just outside the acceptable range of 260 – 320 mOsm/kg, however this very small deviation is not considered to have influenced the outcome of the present study. The pH was found to be within the acceptable range of 7.4 – 7.7.

4.3 The positive control

The Sodium Dodecyl Sulphate (SDS) stock solution 1200 µg/mL, was diluted in growth medium to obtain concentrations of 120, 100, and 80 µg/mL and used as a positive control substance. The stock solution was sterilized by filtration and stored at 4°C for no more than 12 months. The solution should obtain room temperature prior to use.

4.4 Test system

The Neutral Red Uptake Assay in the present study is performed according to the principles given in *INVITTOX* protocol No. 46, "BALB/C 3T3 Cytotoxicity Test". *INVITTOX*, a database on test method protocols created in 1989, was originally based at the Fund for the Replacement of Animals in Medical Experiments (FRAME, UK) and sponsored by the European Commission and the European Research Group for Alternatives Toxicity Testing (ERGATT). With the establishment

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of SIS, Scientific Information Service, in 1996, the responsibility for *INVITTOX* has been taken over by ECVAM, the European Centre for the Validation of Alternative methods.

The present test system is an established mouse fibroblast cell line L929. It was received from Imperial Laboratories, UK, on the 8 September 1999, lot number 057777, identified as passage No 567. The cells were shipped in liquid medium and immediately grown in Ham's growth medium from the supplier. Thereafter the cells were grown in EMEM with 10% FBS at 37°C and 5% CO_2. When the culture was confluent it was trypsinated and the cells were stored in liquid N_2 as a first NZ subculture cell master. To decrease the temperature at which ice is formed, 8-10% DMSO was added as cryoprotective agent.

From the first subculture master the first passage use-culture was made.

After approximately 20 passages, a new use-culture from the freezer was taken into use. The cells were thawed rapidly in a water bath at 37°C and put into growth medium. The DMSO is removed by centrifuging for 5 min at 200 *g* and the deposit of cells was suspended in medium again. The cell suspension was then incubated in a culture flash at 37°C and 5% CO_2.
The present cell culture was passage number 574, i.e 7 passages after the cell culture was received from the supplier.

5. METHODS

The test was performed according to standard method SAT-SM-0010.01, version 05. L929 fibroblasts were grown in EMEM medium with 10% FBS in 96-well micro-plates, 150 µL (5×10^5 cells per mL) cell culture was added per well. Plates were incubated for 24 hours at 37°C, establishing a near confluent monolayer. 150 µL test material dilutions in medium were added (at least 4 replicates) and incubated for 24 hours at 37°C. The cell morphology was checked before and after addition of test material. One extra 96-well plate was used to determine the cell viability at time= 0, before the addition of the test material, using the Neutral Red assay as described below.

The test material was replaced by 150 µL Neutral Red solution. Plates were in the CO_2 incubator for 3 hours at 37°C, thereafter washed twice in medium without FBS, before 100 µL Neutral Red desorb solution was added to each well to leach the stain from the cells. The plates were agitated well to evenly distribute the released Neutral Red, and the absorbance at 540 nm (OD_{540}) of each well was measured to indicate the number of cells surviving exposure to the test materials. The concentration of the test substance required to reduce the viability of the treated test system to 50% of that of the untreated control test system was determined as the endpoint (NRU_{50}) by extrapolation from the dose-response curve.
Dosing:
The following concentrations were selected for the Pullulanase, batch PPY 25645, in the present test model:

30, 10, 3, 1 and 0.3 mg/mL per mL growth medium (EMEM, 10% FBS).
Positive control: 120, 100, 80 µg SDS per mL growth medium.

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Appropriate solvent control wells (untreated control cells) and blank controls (solvent alone without cells) were included on the micro-plate.

6. RESULTS AND DISCUSSION

The basis of the present test system is that the uptake of Neutral Red into the lysosomes of living cells is a good end-point of cytoplasmic membrane integrity. The test can be used as a rapid screening bioassay in a first order *in vitro* battery which can provide an indication of toxicity, related to the concentration of the test substance.

The results obtained in this investigation appear from table 1 and Fig 1.

Table 1:

Test substance	Concentration mg/mL	Viability %	NRU_{50} mg/mL
Pullulanase, batch PPY 25645	0	100	~18
	0.3	98	
	1	99	
	3	101	
	10	85	
	30	5	
Positive control	**Concentration µg/mL**	**Viability %**	**NRU_{50} µg/mL**
SDS	0	100	92
	80	81	
	100	22	
	120	1	

The cell viability of untreated control cells in medium at time 0 and 24 hrs. was estimated, showing that the number of control L929 cells had increased 2.8 x over the exposure period, thereby confirming that the cells were in growth phase. The average doubling time for fibroblasts in cell culture flasks is known to be approx. 24 hrs.

The concentration of the test substance required to reduce the viability of the treated test system to 50% of that of the untreated control test system was determined as the endpoint (NRU_{50}) by extrapolation from the dose-response curve.
The estimated NRU_{50} values for Pullulanase, batch PPY 25645, and the positive control, SDS, are given in table 1.

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Fig.1

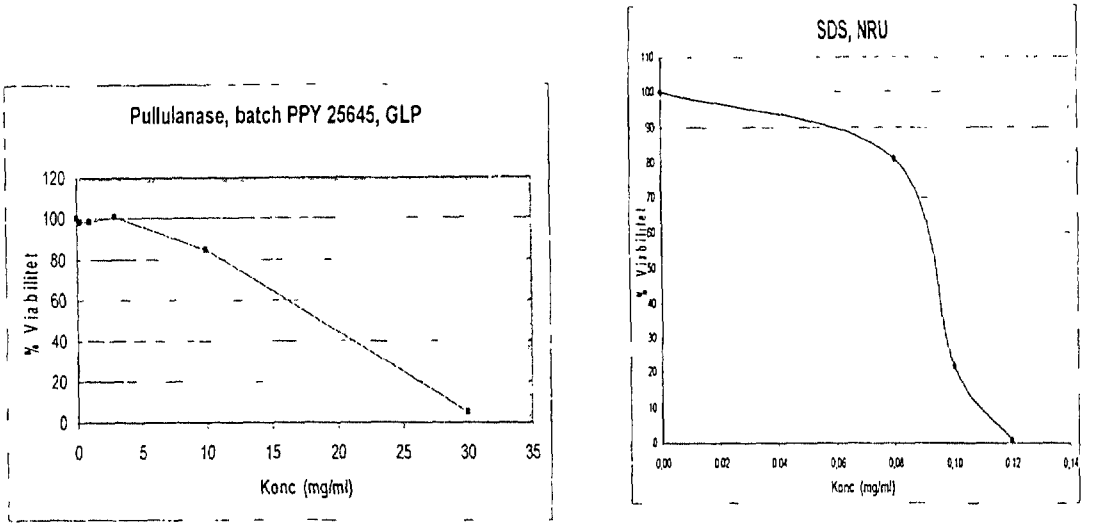

Fig 1. The concentration-response cytotoxicity curves for Pullulanase, batch PPY 25645 and SDS, used, if feasible, to estimate the mid-point cytotoxicity value, NRU_{50}.
The NRU_{50} value for Pullulanase, batch PPY 25645 was estimated to be approximately 18 mg/ml in the present study representing a non-cytotoxic level applying the mouse fibroblast cell line L929 as test system.
The positive control, SDS, gave a NRU_{50} value of 92 µg/mL. The acceptance criteria is set to be within 2 standard deviations of the historical mean (97 ± 6.0µg/mL) thereby meeting the acceptance criteria of a valid test. The estimated NRU_{50} value of SDS is fully in line with the results given in the reference, *INVITTOX* protocol No. 46.

7. CONCLUSION

The results show, that Pullulanase, batch PPY 25645 is non-cytotoxic, in the present *in vitro* Neutral Red Uptake assay applying the mouse fibroblast cell line L929 as test system.

8. DATA STORAGE

Report, protocol and the raw data or exact copies thereof are filed in the QM Central Archive, Bagsvaerd.

9. REFERENCES

(1) *INVITTOX* protocol No. 46, "BALB/C 3T3 Cytotoxicity Test", Jan. 1992 (available electronically from SIS, Scientific Information Service, at ECVAM's website)

SUBMISSION END

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Pages	*Author*	*Title*	*Publish Date*	*Publisher*	*BIB_Info*
000026 - 000039	Pariza, Michael W.; Johnson, Eric A.	Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century	April 12, 2001	Regulatory Toxicology and Pharmacology	Volume 33, pgs 173-186
000041 - 000044	de Boer, Anne Sietske; Diderichsen, Borge	On the safety of Bacillus subtilis and B. amyloliquefaciens: a review	1991	Appl Microbiol Biotechnol	Volume 36, pgs 1 - 4

NA- Not applicable