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ORIGINAL SUBMISSION

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April 19, 1999

1999 APR 22 PM 12:57

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

Re: GRAS Notification - Exemption Claim

Dear Dr. Rulis:

Pursuant to proposed 21 C.F.R. § 170.36(c)(1), Enzyme Bio-Systems Ltd. hereby claims that pullulanase derived from a pure culture of a nonpathogenic, nontoxigenic, and asporogenic strain of *Bacillus subtilis* B1-163/pEB301 is Generally Recognized As Safe ("GRAS") and, therefore, is exempt from statutory premarket approval requirements.

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

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

Enzyme Bio-Systems Ltd.
2600 Kennedy Drive
Beloit, Wisconsin 53511-3992

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

Pullulanase enzyme or debranching enzyme derived from a nonpathogenic, nontoxigenic, and asporogenic strain of *Bacillus subtilis* carrying a gene coding for pullulanase from *Bacillus naganoensis*.

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

The above described pullulanase is to be used to hydrolyze starch and starch-related compounds. The enzyme is to be used at minimum levels necessary to achieve the desired effect and according to the requirements for normal production following Good Manufacturing Practices.

[redacted]

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 that 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. In addition to the printed copy, I have included a floppy disk containing an electronic copy of the evaluation. The electronic copy is in two formats of Microsoft Word. The file "EBw97.doc" is in a Word 97/98 format while the file "EBw95.doc" is in a Word 6/95 format. 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.

Very truly yours,

W. Martin Teague
Vice President

Enclosures (2)

cc: M. J. Friesema

A Pullulanase Enzyme Produced by *Bacillus subtilis* B1-163/pEB301

by
Enzyme Bio-Systems Ltd.
2600 Kennedy Drive
Beloit, Wisconsin 53511-3992
USA

[redacted]

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GENERAL INTRODUCTION

Pullulanase, derived from a pure culture of a nonpathogenic, nontoxigenic, asporogenic strain of *Bacillus subtilis* B1-163/pEB301, will be used to hydrolyze starch and starch-related compounds. *Bacillus subtilis* B1-163/pEB301 is a recombinant strain that has been modified to express the pullulanase gene derived from a nonpathogenic, nontoxigenic source organism, *Bacillus naganoensis.*

Starch is a branched polysaccharide composed mainly of linear chains of 1,4 *alpha*-linked D-glucose residues, joined together by 1,6 *alpha*-linkages. Pullulanase cleaves specifically at the 1,6 *alpha*-glucosidic bonds, which is a process called debranching.

Pullulanase can be used in the starch industry for the manufacture of starch hydrolysis products; for example, pullulanase can be used to improve the efficiency of the saccharification process in the production of corn syrups and dextrose. Pullulanase also has value in other industries such as the baking industry, where the enzyme can be used to modify starch in bakery products, or in the alcoholic beverage industry, where pullulanase can be used to increase the amount of fermentable sugars available for conversion into alcohol.

A safety evaluation of pullulanase from *B. subtilis* B1-163/pEB301 was performed that involved an examination of the production organism and the introduced genetic material, a review of published and unpublished literature, and safety testing of both the donor and recipient microorganisms, as well as enzyme product made from this strain.

The purpose of this document is to notify the U. S. Food and Drug Administration that sufficient and valid information has been obtained to demonstrate the Generally Recognized as Safe status of pullulanase from *B. subtilis* B1-163/pEB301.

[redacted]

A. Description of the Substance

1. Source

The pullulanase enzyme product is derived from a nonpathogenic, nontoxigenic, and asporogenic *Bacillus subtilis* designated strain B1-163/pEB301. This strain contains a plasmid that encodes the pullulanase gene from a nonpathogenic, nontoxigenic source organism *Bacillus naganoensis.*

2. Chemical Name

According to the Nomenclature Committee of the International Union of Biochemistry, pullulanase has the following designation: EC 3.2.1.41.

3. Common Or Usual Name

Pullulanase or debranching enzyme

4. Chemical Abstract Service (CAS) Registry Number

The CAS Registry Number for pullulanase is 9075-68-7.

5. Properties

The enzyme preparations are brown liquids.

6. Specifications

Specifications for the pullulanase enzyme preparations meet or exceed the specifications for enzyme preparations as set forth in the Food Chemicals Codex, Fourth Edition (National Academy of Sciences 1996), which is incorporated herein by reference. Furthermore, the enzyme preparations contain no antibiotics or mycotoxins.

[redacted]

B. Manufacturing Process

Pariza and Foster (1983) propose that, when assessing the safety of enzymes used in food processing, there are only two sources of possible toxic contaminants: the enzyme source organism and the manufacturing process. If current Good Manufacturing Practices (cGMP) are used in production, then the manufacturing process can be eliminated as a source for toxic contamination. Enzyme Bio-Systems Ltd. employs a manufacturing process that is common for the industry for production of food enzymes from microorganisms (Pariza and Foster 1983) and consistently abides by cGMP.

1. Strain Preservation

The manufacturing process begins with a pure stock culture of a nonpathogenic, nontoxigenic, asporogenic strain of *B. subtilis* B1-163/pEB301, which is monitored for identity, absence of foreign microorganisms, and enzyme generating ability. Stock cultures are portioned into sterile cryophile ampules, and the ampules are stored at -70° C.

2. Fermentation

For production of the enzyme, the contents of an ampule are transferred aseptically to a seed flask containing a sterile medium. After appropriate incubation, the purity of the flask culture is checked using microscopic examination. If contamination has occurred, the culture is destroyed. If pure, the contents of the flask are transferred aseptically to a seed fermentor, incubated under appropriate conditions, and again tested for purity. The second pure culture is transferred to a production fermentor where commercial quantities of the enzyme are produced.

The sterile medium in the production fermentor contains a combination of the following:

soy flour
dextrose
yeast extract
magnesium sulfate
potassium phosphate
calcium chloride
manganese chloride
antifoam [1]
    polypropylene glycol
    dimethylpolysiloxane

1. The antifoams are listed as processing aids used by the enzyme industry in the letter to Dr. Alan Rulis from the Enzyme Technical Association dated December 20, 1996.

These ingredients, in aggregate, comprise approximately 10% by weight of the fermentor medium. The remainder is potable water. All

[redacted]

ingredients either meet FCC specifications or are "food grade."

A separate fermentor or similar sterilizable tank contains a supplementary feed supply that is sterilized and fed into the fermentor during enzyme production.

The fermentation used to grow the *B. subtilis* B1-163/pEB301 organism is an aerobic, submerged, pure culture fermentation that is carried out in closed stainless steel vessels equipped with agitators. During fermentation, the growth of the organism and the enzyme production rate are monitored by measurements of parameters such as temperature, pH, aeration rate, dissolved oxygen, enzyme activity analysis, culture purity, assay of nutrient levels, etc. Should evidence of a significant contamination exist, the fermentor and its contents are sterilized by heating, then the contents are discarded.

3. Recovery and Stabilization

When a fermentation is completed, the broth is clarified using rotary vacuum filtration through a filter medium of diatomaceous earth. The enzyme in the clear filtrate is then concentrated using membrane ultrafiltration to the desired activity level. The food approved sodium benzoate and potassium sorbate are added to extend the shelf-life of the pullulanase product. The product contains no viable production organisms.

[redacted]

C. Use of the Substance

1. Foods In Which Used And Purpose Of Use

a) Starch Hydrolysis Products

The bacterial pullulanase derived from *B. subtilis* B1-163/pEB301 will be used in a way similar to other debranching enzymes, that is, in the hydrolysis of starch or starch related compounds, particularly in the production of corn sweeteners.

In general, corn sweeteners are made using a series of enzymes to catalyze the hydrolysis of starch. First, a bacterial *alpha*-amylase can be used to thin gelatinized starch. Additional enzymes, including glucoamylase, *beta*-amylase, or maltogenic amylase, are used to saccharify the thinned starch into higher dextrose equivalent syrups. Although glucoamylase does possess debranching capability, the reaction is relatively slow and there are substrate size requirements. Pullulanase can be added during the saccharification process to further enhance the saccharide yield. Other advantages that can be seen from the addition of pullulanase include a faster reaction time and a reduction in the amounts of saccharifying enzyme needed. In the case where glucoamylase is used, the refined hydrolysate may be treated with another enzyme, called glucose isomerase, to make high fructose corn syrup (HFCS).

From the above description, it can be seen that pullulanase can be used to make all types of corn sweeteners.

b) Bakery Products

Pullulanase can also be used in the preparation of baked goods to modify starch in the bakery product. The enzyme is added as a liquid alone or in combination with other enzymes. Addition of the enzyme is made in the baking process at one of several points, depending on the process and the product. The enzyme is added either to the sponge or dough in a sponge-dough process or the dough in a straight dough process, no-time dough, or other process. Alternatively, the enzyme can be added as a liquid slurry with other baking ingredients after mixing. The enzyme can also be premixed with other baking ingredients and added as a part of a standard product containing dough conditioners, dough strengtheners, oxidizing agents, and the like.

c) Alcoholic Beverages

Another use of pullulanase is in the production of alcoholic beverages. Starch hydrolysis provides an increased level of fermentable sugars, which may result in reduced total carbohydrate levels and increased ethanol yields.

[redacted]

2. Level Of Use

It is recommended that the enzyme preparation be used in the following dosages to achieve the intended technical effect:

a) Starch Hydrolysis Products -- 0.0225 to 0.8 U/g ds starch

b) Bakery Products -- 0.0225 to 10 U/g ds flour

c) Alcoholic Beverages -- 0.0225 to 0.8 U/g ds starch

3. Fate Of The Enzyme In Food

The enzyme preparation, net of water, may be represented by its total solids level. Total solids include ash constituents that can be considered inert relative to safety issues. Non-ash components of the product are measured as total organic solids (TOS), which include residue from the fermentation and metabolic end products from the production microorganism. The TOS include enzyme protein, the functional agent of the preparation. Enzyme residues are presented as estimated TOS per unit of enzyme in commercial product.

Total protein comprises about 58% of the TOS, of which about 7.2% is enzyme protein. Thus, one can use the levels of introduced protein or enzyme protein combined with other known factors to estimate the residue levels. We cannot use the known relationship between activity and enzyme protein to determine the TOS since the enzyme denatures in use.

a) Starch Hydrolysis Products

One intended use of the enzyme is in the production of many different types of starch hydrolysates. These products may be treated further; for example, a glucose syrup may be isomerized to make HFCS, then refined to achieve any one of several possible commercial products. As stated above, the dosage range for starch hydrolysis products is from 0.0225 to 0.8 U/g ds, which is equivalent to a maximum TOS introduction of 1.9×10^{-5} g/g of starch ds. The hydrolysate contains no detectable active pullulanase activity as measured by the standard method or by the enzyme-linked immunosorbent assay (ELISA) method. The limit of detection for pullulanase using the ELISA method is 2.8×10^{-4} µg/g ds starch, a level that represents 0.07% of the introduced material.

The conservative estimate of TOS residue from enzyme product contained in the starch hydrolysis products is 1.3×10^{-8} g of TOS per g product ds.

b) Bakery Products

For bakery products, the added material is not removed by further refining. Using a maximum recommended dosage of 10 U/g of

 [redacted]

flour, the TOS from pullulanase product in the bakery product would be 2.4×10^{-4} g of TOS per g of flour.

c) Alcoholic Beverages

For alcoholic beverages, such as beer or distilled spirits, residual protein can be calculated in a manner similar to the presentation for starch hydrolysis products. In the manufacture of beer, the feed stock treated with the pullulanase enzyme is, on average, about 14% ds. We make the simplifying and conservative assumption that the dissolved solids are all starch. Using a dosage of 0.0225 to 0.8 U/g of starch ds, the maximum TOS introduced into the beer would be 2.7×10^{-6} g of TOS per ml. It has been shown that about 96% of the initial total protein entering beer production is removed during manufacture (Broderick 1977). Assuming a similar amount of enzyme protein would be removed, one calculates that the maximum enzyme product TOS residue found in beer would be 1.1×10^{-7} g/ml.

For distilled spirits, the initial dry substance is 3800 g per proof gallon, and the distillation process removes at least 99% of the protein residue. The maximum TOS contributed by the pullulanase product in the alcoholic beverage would be 7.2×10^{-4} g per proof gallon.

D. Intended Technical Effect

Pullulanase derived from *B. subtilis* B1-163/pEB301 will be used to hydrolyze the 1,6 *alpha*-glucosidic bonds of starch and other starch-related compounds.

[redacted]

E. General Recognition of Safety

1. Production Organism

Pariza and Foster (1983) have reported that the safety of the production organism should be the prime consideration when assessing the safety of an enzyme intended for food use. For recombinant DNA organisms, it has been suggested that the safety of the component parts of the production strain be evaluated (i.e., the host organism, the DNA insert from the donor organism, and the vector(s) used) when assessing safety (Andersen *et al.* 1987, International Food Biotechnology Council 1990, OECD 1986, Scientific Committee for Food 1992). The production organism and its component parts are discussed below.

The pullulanase of this document is derived from a nonpathogenic, nontoxigenic, asporogenic strain of *B. subtilis* designated strain B1-163/pEB301. This strain is a recombinant strain that has been modified to express the pullulanase gene from *B. naganoensis. Bacillus naganoensis* is a recently described species (Tomimura *et al.* 1990) that was isolated from a hot spring. The type culture is on deposit at the American Type Culture Collection (ATCC) as strain ATCC 53909 and is available to the public.

a) Recipient Microorganism

The recipient microorganism, strain B1-163, used in construction of the production organism is derived from a well known and widely used strain of *B. subtilis* designated strain 168. Strain 168 was genetically altered through a series of transformations using DNA from other *B. subtilis* strains to form strain B1-109. This strain has a long history of safe use in enzyme production and is the subject of two GRAS Affirmation Petitions (CPC International, Inc. 1986, Enzyme Bio-Systems 1988). Classical mutation and selection techniques were performed using strain B1-109 to generate the low protease-producing strain B1-163. A detailed characterization of strain B1-163 is given in Appendix 1. A fatty acid profile comparison test of strains B1-109 and B1-163 was performed by Microbial ID, Inc. (Newark, DE). The results (see Appendix 2) show the two strains are the same subspecies or biotype of *B. subtilis*.

The recipient microorganism contains only *B. subtilis* DNA and characteristically does not require aromatic amino acids for growth, is asporogenic, and does not produce pullulanase. These traits are stable in this strain. For example, during repeated attempts to determine the frequency of reversion to a sporogenic strain, no spores have ever been found (CPC International, Inc. 1986). Reversion to a sporogenic strain is estimated to be less than one in 100 million.

[redacted]

Bacillus subtilis is a common saprophyte that inhabits soil and is thought to contribute to nutrient recycling due to the variety of enzymes that members of the species are capable of producing. *Bacillus subtilis* is also commonly found as a contaminant in food and on the skin of animals and humans. The organism including various genetically modified strains are listed in the Food Chemicals Codex as a source of carbohydrase and protease enzyme (National Academy of Sciences 1996). *Bacillus subtilis* is the subject of the FDA GRAS Affirmation petition 3G0016 and has a long history of safe use in food enzyme manufacture. Furthermore, *B. subtilis* is the type species for the genus and is not considered to be a pathogen. The species, in general, is often represented as the exemplar of nonpathogenic microorganisms.

b) Introduced Genetic Material

(1) Limited in size

The vector used to introduce the pullulanase gene from *B. naganoensis* ATCC 53909 into *B. subtilis* B1-163 is a plasmid designated pEB301. The details of the construction of this plasmid are given in Appendix 3. Plasmid pEB301 is composed of a 2,973 base pair portion of DNA containing the *B. naganoensis* pullulanase gene, a 3,594 base pair portion of DNA from plasmid pUB110, and a 777 base pair portion from plasmid pCPC717. Each of these portions is discussed individually below:

The 2,973 base pair portion of plasmid DNA that originated from *B. naganoensis* includes the genetic material that codes for the pullulanase gene.

The plasmid pUB110 was used to subclone the *B. naganoensis* pullulanase gene into *B. subtilis.* This plasmid has been used for many years as a cloning vehicle for transforming *B. subtilis*, is approved for use by the National Institutes of Health, and has a long history of safe use as a cloning vector in the production of food enzymes from recombinant organisms (International Food Biotechnology Council 1990).

The DNA sequence data of plasmid pUB110 indicate that this plasmid contains five open reading frames (ORF) (McKenzie *et al.* 1986). The ORF are designated:

(1) *alpha* - a region coding for an essential DNA binding protein thought to be involved in plasmid replication,

[redacted]

(2) *beta* - a region which produces in minicells a 58,000 dalton protein of unknown function,

(3) *delta* - a short ORF of unknown function that overlaps the *beta* ORF,

(4) *gamma* - a 11,000 dalton ORF which encodes bleomycin resistance, and

(5) neo^r - an ORF which produces in minicells a 38,000 dalton protein responsible for kanamycin resistance.

In plasmid EB301, most of the *beta* region has been deleted. Both the *gamma* and neo^r regions remain in plasmid pEB301 and cells containing this plasmid are both kanamycin and bleomycin resistant. Antibiotic resistance was used as a selection marker during the cloning of the *B. naganoensis* pullulanase gene. Because the recombinant DNA production organism does not enter the food product nor is it deliberately released to the environment, the presence of these antibiotic resistance genes is not a concern (International Food Biotechnology Council 1990, Food and Drug Administration 1998).

No products from *alpha* or *delta* ORF have been observed in minicells.

Plasmid EB301 also contains a 777 base pair portion of DNA that originated from the plasmid pCPC717. Plasmid pCPC717 is a *Bacillus stearothermophilus alpha*-amylase encoding plasmid used in the construction of plasmid pCPC720. Plasmid pCPC720 was the vector transformed into *B. subtilis* for use in the production of the *alpha*-amylase preparation that is the subject of the Generally Recognized as Safe (GRAS) petition 4G0293 (CPC International, Inc. 1986). The portion of plasmid pCPC717 that is contained in plasmid pEB301 originated from *B. stearothermophilus* and contains the promoter and terminating regions of the *alpha*-amylase gene.

In summary, plasmid pEB301 is limited in size and consists only of the pullulanase structural gene of interest, regulatory sequences permitting the expression of the pullulanase gene, and associated nucleotide sequences needed for transfer and maintenance.

The derivation of pEB301 and subsequent transformation of pEB301 into *B. subtilis* B1-163 was performed once and is not repeated each time a production strain is needed. The production strain *B. subtilis* B1-163/pEB301 is stable and is

stored under conditions such that it can be retrieved for pullulanase production when needed.

(2) Poorly Mobilizable

The ability of the introduced genetic material to be transferred and mobilized is inactivated. The frequency of transfer is less than 10^{-8} transfer events per recipient (see Appendix 4).

2. Literature

a) Literature Review

An extensive search of the published literature keyed to the enzyme and the donor microorganism used for construction of the production organism was performed. The search strategy covered the period 1957 to the present and used synonyms for both the organism and the enzyme.

A review of the literature search on the donor organism uncovered no reports that implicate *B. naganoensis* in any way with a disease situation. Indeed, because *B. naganoensis* is a newly described organism (Tomimura *et al.* 1990), it is not surprising that few references to the organism were found.

The literature search on the enzyme was restricted to those databases that addressed health or safety concerns. No reports indicating that pullulanase poses a human health concern, and no adverse data were uncovered.

In light of the general acceptance of the safety of *B. subtilis* as a species and our specific long history of safe use of strain B1-163 in the production of food enzymes, a review of the literature of *B. subtilis* is not warranted. The safety of *B. subtilis* is addressed in a recent review (de Boer and Diderichsen 1991).

b) Published Literature to Support GRAS Status

The nature and ubiquity of enzymes are well known and documented (Dixon and Webb 1979). Their application to food processing has developed from traditional recipes such as those from fermented foods whose preparation is older than recorded history. The enzymes used today in modern food processing are obtained from animal and plant tissues and microorganisms.

From a historical perspective, the use of microorganisms as a source of enzymes used in food processing is relatively recent. The development of food processing enzymes is a result of technological and economic demands fostered by the food industry. Today, microorganisms are the major source of food grade enzyme preparations. Zeman and Teague (1992) list 57

food grade enzyme preparations that are classified according to source and activity. Of these, 70% are obtained from microbial sources; thus, the safe use of enzymes, and microbial enzymes, in particular, is well known in the food processing arena.

Pullulanase is a naturally occurring enzyme activity in many microbial species. The National Research Council/National Academy of Sciences reported that pullulanase was first used in 1974 in the United States for production of corn syrup, high fructose corn syrup (HFCS), and dextrose production (1981).

Enzymes are used in foods to accomplish a variety of purposes. A major use of enzymes is for the hydrolysis of starch. Starch hydrolyzing enzymes are generally called carbohydrases and, more specifically, amylases. With few exceptions, all the amylase preparations available to the food industry are obtained from microbial sources. Amylase preparations from molds generally contain the amylases *alpha*-amylase and glucoamylase, while bacterial strains are usually used to produce *alpha*-amylase or pullulanase. Many amylases from both molds and bacteria have a long history of safe use in foods.

The glucoamylase from several *Aspergillus* species is an enzyme that possesses debranching activity (as well as *alpha*-1,4-glucosidic activity) and has long been used for the hydrolysis of starch. In 1973, The Ad Hoc Enzyme Technical Committee, which is now called the Enzyme Technical Association, filed a petition seeking GRAS affirmation from the U.S. Food and Drug Administration (FDA) for a number of enzymes, including the glucoamylase from two *Aspergillus* species. This petition, designated GRASP 3G0016, asks for GRAS affirmation based on a history of common use prior to the passage of the Food Additive Amendment to the Food and Drug Act in 1958. Although the petition is still pending, glucoamylase is marketed in the United States and its GRAS status has not been disputed by the FDA.

In 1985, a GRAS affirmation petition submitted to the FDA for a pullulanase from *B. acidopullulyticus* was accepted for filing (Novo Laboratories Inc. 1985). In 1995, a petition proposing that the pullulanase enzyme preparation derived from *B. licheniformis* containing the pullulanase gene from *B. deramificans* be affirmed as GRAS was accepted for filing (Solvay Enzymes, Inc. 1995) and a safety evaluation of this pullulanase has been published (Modderman and Foley 1995). Although FDA regulations for these enzymes have not yet issued, they are widely used throughout the world and use of these enzymes has generated no known safety concerns.

Pariza and Foster (1983) have reported that the safety of the

production organism should be the prime consideration when assessing the safety of an enzyme intended for food use. For rDNA organisms, it has been suggested that the safety of the component parts of the production strain be evaluated (i.e., the host organism, the DNA insert from the donor organism, and the vector(s) used) when assessing safety (Andersen *et al.* 1987, International Food Biotechnology Council 1990, OECD 1986, Scientific Committee for Food 1992).

As discussed previously, *B. subtilis* is used widely as a safe host for production of many important industrial enzymes (de Boer and Diderichsen 1991, National Academy of Sciences 1996). The specific strain B1-163 is a first generation mutant of strain B1-109. Strain B1-109 was genetically modified by transformation with either plasmid pCPC720, which encodes an alpha-amylase gene from *B. stearothermophilus*, or plasmid pCPC800, which encodes an alpha-amylase gene from *B. megaterium*. This strain has been used for over ten years in the manufacture of these two enzymes (CPC International, Inc. 1986, Enzyme Bio-Systems Ltd. 1988, Zeman and McCrea 1985, Zeman and Teague 1992). The subchronic toxicity of enzyme product from the two recombinant *B. subtilis* B1-109 strains was tested in two different animal species and found to produce no toxicological effects in either animal model at the tested dose levels (MacKenzie *et al.* 1989a and b).

Furthermore, the plasmid pEB301 is nearly identical to the plasmids pCPC720 and pCPC800 (CPC International, Inc. 1986, Enzyme Bio-Systems Ltd. 1988) which have been used for at least 10 years to make food-grade amylases. All three plasmids are composed mainly of DNA derived from plasmid pUB110, a plasmid with a documented prior safe use in the preparation of a food product (International Food Biotechnology Council 1990) - the major difference in the three plasmids being the carbohydrase gene of interest.

Finally, the donor organism from which the pullulanase gene was obtained, *B. naganoensis*, has been tested for virulence potential and was shown to be nonpathogenic and nontoxigenic in mice (Tomimura *et al.* 1990), thus one can assume the pullulanase gene is safe.

In summary, pullulanase is known to be safe and has been used for a number of years in the food industry, the production host for manufacture of the pullulanase has a documented history of safe use, enzyme product made from the parent host of strain B1-163 has been shown to lack subchronic toxicity, the donor organism is nonpathogenic and nontoxigenic, and the vector used has a reported history of safe use in the food industry, thus the GRAS

status of pullulanase from *B. subtilis* B1-163/pEB301 is supported by the published literature. This published information is sufficient for determination of pullulanase from *B. subtilis* B1-163/pEB301 to be GRAS based on scientific information by experts in the field.

3. Additional Safety Testing

Pariza and Foster (1983) offer that, if there are no safety issues involving the production organism, then the only other source of possible toxic contaminants is the manufacturing process. The following safety tests demonstrate the safety of the final product, and thus show that there is no toxic contaminant introduction during manufacturing of the enzyme.

a) Recipient Microorganism

The recipient microorganism, strain B1-163, used in the construction of the production microorganism, has been tested previously for virulence potential in mice. When tested, the strain contained plasmid pCPC800, a plasmid encoding for an amylase commonly used in food. The microorganism was found to be nonpathogenic and nontoxigenic under the conditions of the test.

b) *Bacillus naganoensis* Pullulanase

The pullulanase from *B. naganoensis* has undergone extensive testing; the results of the safety studies using this enzyme are summarized here.

(1) Virulence Testing

The *B. naganoensis* organism was tested for virulence potential by injection into mice. No animals died on test and no abnormalities were seen at necropsy. It was concluded that the organism was nonpathogenic and nontoxigenic in mice under the conditions of the test. These results were published in the initial article describing the new species (Tomimura *et al.* 1990).

(2) 14-Day Acute Oral Toxicity In Rats

An acute oral toxicity study, using young adult albino rats as test animals, was conducted. The animals were given up to 5000 mg/kg body weight of the test material administered by oral gavage. The results of this test demonstrated the absence of toxic substances in pullulanase product from *B. naganoensis*.

A summary of this study is included as Appendix 5.

(3) Mutagenicity Studies

(a) Salmonella/Mammalian-Microsome Reverse

[redacted]

Mutation Assay or Ames Test

The mutagenic activity of the *B. naganoensis* pullulanase product was tested using the Ames test. This assay evaluated the test article for its ability in induce reverse mutations at the histidine locus in the genome of specific *Salmonella typhimurium* tester strains both in the presence and the absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor™-induced rat liver (S9). The results of the Ames test indicate that, under the conditions of the study, the pullulanase product did not cause a positive increase in the number of histidine revertants per plate of any of the tester strains either in the presence or absence of microsomal enzyme.

Results are tabulated in Appendix 6.

(b) Micronucleus Assay

This *in vitro* assay evaluated the ability of the *B. naganoensis* pullulanase product to induce micronuclei in bone marrow polychromatic erythrocytes of CD-1 (ICR) mice. The results showed that the test material did not induce a significant increase in micronuclei in the erythrocytes under the conditions of this assay and is considered negative in the mouse bone marrow micronucleus test.

Results are tabulated in Appendix 6.

(c) Forward Mutation Assay

The objective of this *in vitro* assay was to evaluate the ability of the *B. naganoensis* pullulanase product to induce forward mutations at the thymidine kinase (TK) locus in the mouse lymphoma L5178Y cell line. The test material was considered to be negative in this activation assay.

Results are tabulated in Appendix 6.

(4) 4-Week Dietary Toxicity Study

The purpose of this study was to determine the potential for short-term toxicity including neurobehavioral toxicity of the pullulanase enzyme preparation when fed to rats for at least four weeks.

It was concluded that the dietary administration of *B. naganoensis* pullulanase to rats for at least four weeks

resulted in no adverse systemic effects. Lower mean cumulative body weights and lower food consumption were noted only on males given the highest dosage of enzyme product and were small in magnitude (7 to 10%) during any one week. These data suggest that the high pullulanase diet was less palatable to the male rats. Overall mean body weights were similar across all groups and there were no adverse clinical findings in the animals fed a diet containing pullulanase enzyme product. The pullulanase product diet had no effect on the neurobehavioral, clinical pathology, or anatomical pathology results.

The no-observable-adverse-effect level of the pullulanase product in the diet is considered to be 0.75% for male rats and 1.50% for female rats.

A summary of this study is included as Appendix 7.

c) *Bacillus subtilis* B1-163/pEB301 Pullulanase

(1) 14-Day Acute Oral Toxicity In Rats

An acute oral toxicity study, using young adult albino rats as test animals, was conducted. The animals were given up to 5000 mg/kg body weight of the test material administered by oral gavage. The results of this test demonstrated the absence of toxic substances in pullulanase product from *B. subtilis* B1-163/pEB301.

A summary of this study is included as Appendix 8.

(2) 4-Week Dietary Toxicity Study

The purpose of this study was to determine the potential for short-term toxicity of the *B. subtilis* B1-163/pEB301 pullulanase enzyme preparation when fed to rats for at least four weeks.

It was concluded that the dietary administration of *B. subtilis* B1-163/pEB301 pullulanase to rats for at least four weeks resulted in no adverse effects at levels up to and including 1.25% dietary, the highest level tested. There were no statistical differences in means of body weight or food consumption between the groups. Mean body weight changes were statistically higher between weeks 4 and 5 for males given 1.25% pullulanase; however, this is not considered to be toxicologically important. The pullulanase product diet had no effect on the clinical pathology or anatomical pathology results.

The no-observable-adverse-effect level of the pullulanase

[redacted]

product in the diet is considered to be 1.25% for male and female rats.

A summary of this study is included as Appendix 9.

4. Consumer Exposure Data

As stated in above, estimates of residue in food are expressed as grams TOS per unit of consumed food. These estimates are used further to estimate consumer exposure data. TOS is composed of residues from the fermentation medium and metabolic end products from the production microorganism, including the functional material, the pullulanase enzyme. Use of TOS excludes consideration of ash.

In this section, a "worst case" estimate of human exposure to the residues of the enzyme product (expressed as g of TOS per kg body weight per day) from all sources is made using consumption and population estimates for the United States.

a) Summary Of Enzyme Residue Intake

The estimate of exposure to enzyme residues, estimated as g of TOS per kg body weight per day, is as follows:

Source	Residue ($x\ 10^{-6}$)
Starch Hydrolysis Products	0.02
Baked Goods	209.27
Beer	0.43
Distilled Spirits	0.05
Total Exposure	209.77

b) Human Consumption Versus No-Effect Level In Feeding Study

The enzyme preparation used in the toxicity studies contained 11.0% ash and 11.4% moisture, which calculates to a TOS of 77.7%. In the 4-week dietary study, rats ate as high as 1,313 mg/kg per day of product. This calculates out to be consumption of 1.02 g of TOS per kg body weight per day; a ratio of feeding study TOS to estimated maximum human consumption of TOS shows that animals in the highest dose group were fed up to about 4860 times greater the amount of enzyme that would be expected to be consumed by humans. In the acute oral toxicity study, rats were exposed to 5000 mg/kg per day of product or 3.88 g TOS per kg body weight. On a daily basis, this is 18509 time greater the amount of enzyme what would be expected to be consumed by humans.

[redacted]

CONCLUSION

Enzyme Bio-Systems Ltd. has evaluated the pullulanase enzyme derived from *B. subtilis* B1-163/pEB301 and determined that this enzyme is GRAS based upon scientific procedures in accordance with 21 C. F. R. 170.30 (b) for use in the hydrolysis of starch and starch-related compounds. This determination is based on an extensive review of published and unpublished safety data available on pullulanases, the production organism (including its component parts), and the pullulanase enzyme product.

[redacted]

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[redacted]

Appendix 1: Characterization of *Bacillus subtilis* Strain B1-109, the Precursor to Host Strain B1-163

A series of tests was performed on *Bacillus subtilis* strain B1-109 to determine strain characteristics. Test selections were based on information in Bergey's Manual (1) and a taxonomic key (2) developed by R. E. Gordon, an authority on *Bacillus* taxonomy.

B. subtilis strain 168 was used as the indicator strain. Inocula for the tests were obtained by growing the strains in tryptic soy broth with glucose (TSBG), tryptic soy agar with 1% starch (TSA/S), or tryptic blood agar base plus 1% starch (TBA/S) unless otherwise noted.

Test conditions were as follows:

1. Anaerobic Growth: Plates of TBA/S were allowed to equilibrate in an anaerobic hood for 3 days. Cultures were streaked on the plates in an anaerobic hood. The inoculated plates were placed in an anaerobic Gas-Pak jar, incubated at 37°C for 3 days, and examined for growth.
2. Catalase Production: Approximately 0.1 ml of hydrogen peroxide was dropped on overnight cultures grown on TBA/S plates. Production of bubbles indicated the presence of catalase.
3. Glucose Utilization: Basal agar medium containing glucose and a pH indicator dye was inoculated with overnight cultures. After 24 hours incubation at 37°C, a shift in the pH due to the production of acid from glucose changes the indicator in the medium from red to yellow.
4. Gram Staining: A drop of an overnight-broth culture was placed on a clean glass microscope slide, allowed to air-dry, heat-fixed, and gram stained.
5. Motility: A wet mount of an overnight culture as examined using phase contrast microscopy. Directional movement of the cells (in contrast to Brownian motion) indicated motility.
6. Propionate Utilization: Propionate agar was made with 2 gm sodium propionate, 1 gm NaCl, 0.2 gm $MgSO_4 \cdot 7H_2O$, 0.5 gm $(NH_4)_2HPO_4$, 15 gm agar, 1000 ml distilled water, and 20 ml of a 0.04% (w/v) solution of phenol red. The pH was adjusted to 6.8 before autoclaving. Slants of this medium were inoculated and incubated at 37°C. Utilization of the propionate was established if the alkaline color or phenol red appeared after 7 days of incubation.
7. Spore Formation, Position, and Morphology: Wet mounts of cultures grown for 3 days on sporulation indicator medium were examined for spores. To further

[redacted]

test for sporulation, the cultures were used to inoculate TSBG. Inoculated broths were then vortexed, heated at 90°C for 10 minutes to kill vegetative cells, incubated at 37°C, and observed for growth.

8. Starch Hydrolysis: Cultures were grown on TBAB/S plates for three days. The plates were flooded with an iodine solution, which binds with starch and produces a blue color. A clear zone underneath and around the growth area indicated the organism produced an amylase that hydrolyzed starch.

9. Tolerance Tests: Growth of the strains under various conditions were tested as follows:

   9.1 Growth at Various Temperatures: TSBG was inoculated with the strains; incubated at 30°C, 37°C, or 50°C for 3 days; and monitored for growth.

   9.2 Growth at pH 5.7: TSBG was adjusted to a pH of 5.7 using HCl before sterilization. The broth was inoculated with the strains, incubated at 37°C for 3 days, and observed for growth.

   9.3 Growth in 7% NaCl: TSGB with 7% (w/v) NaCl was inoculated, incubated for 3 days at 37°C, and examined for growth.

10. Voges-Proskauer Test: Voges-Proskauer medium with glucose was inoculated with the strains and incubated at 37°C overnight. Production of acetoin was detected by the addition of two drops of a 20% (w/v) aqueous solution containing 0.3% (w/v) creatine and three drops of a 5% (w/v) solution of *alpha*-napthol in absolute ethyl alcohol. The presence of acetoin was indicated by the development of a red color within 20 minutes.

Results of the above tests are presented in Table 1.

Cultures of *B. subtilis* 168 and B1-109 gave identical results in a series of carefully chosen tests with two exceptions. Strain B1-109 did not hydrolyze starch or form spores. These results were as expected.

References

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[redacted]

TABLE 1. Characterization of *Bacillus subtilis* strain B1-109

Test		*B. subtilis Strain* 168	B1-109
1.	Anaerobic Growth	-	-
2.	Catalase Production	+	+
3.	Glucose Utilization	+	+
4.	Gram Stain	+	+
5.	Motility	+	+
6.	Propionate Utilization	-	-
7.	Spore Characterization		
	(a) Formation	+	-
	(b) Shape	Ellipsoidal	
	(c) Dominant Position	Central	
8.	Starch Hydrolysis	+	-
9.	Tolerance Tests		
	(a) 30°C	+	+
	(b) 37°C	+	+
	(c) 50°C	+	+
	(d) pH 5.7	+	+
	(e) 7% NaCl	+	+
10.	Voges-Proskauer Test	+	+

[redacted]

Appendix 2: Characterization of *Bacillus subtilis* Strain B1-163, the Low Protease Variant of B1-109 and Host for the Pullulanase-containing Plasmid pEB301

Bacillus subtilis strains of B1-109 and B1-163 were compared using fatty acid analyses. This comparison was done by Microbial ID, Inc. (115 Barksdale Professional Center, Newark, Delaware 19711 USA). The following is a representation of the results. The original is on file and available. The results show that the two strains are the same bio-type or sub-species of *B. subtilis*. Within the data, the sample mark of "EBS-1" refers to *B. subtilis* strain B1-109 while the mark "EBS-2" refers to *B. subtilis* strain B1-163.

[redacted]

MICROBIAL ID, INC.
115 BARKSDALE PROFESSIONAL CENTER
NEWARK, DE 19711 (302) 737-4297

DATE MAY 27, 1992

TO JEFF FRANKIEWICZ
ENZYME BIO-SYSTEMS, LTD.
ARLINGTON HEIGHTS, IL 60005

SUBJECT FA ANALYSIS and INFO

ENCLOSED ARE YOUR PROFILES and CHROMATOGRAMS. YOUR SAMPLES NAMED CONCLUSIVELY. I ALSO PERFORMED A DENDROGRAM ANALYSIS TO NOTE ANY SIMILARITIES BETWEEN THEM. I'VE SENT SOME LITERATURE ALONG THAT DESCRIBES OUR SYSTEM AND PROGRAMS. PLEASE FEEL FREE TO CALL IF YOU HAVE QUESTIONS. THANKS FOR YOUR INTEREST IN MIDI.

SIGNED

[redacted]

BOTTLE: 15 ID# 1491 WED 27-May-92 00:19:07

FILE DATA: F92526545

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RUN # 48 MAY 27, 1992 16:55:40

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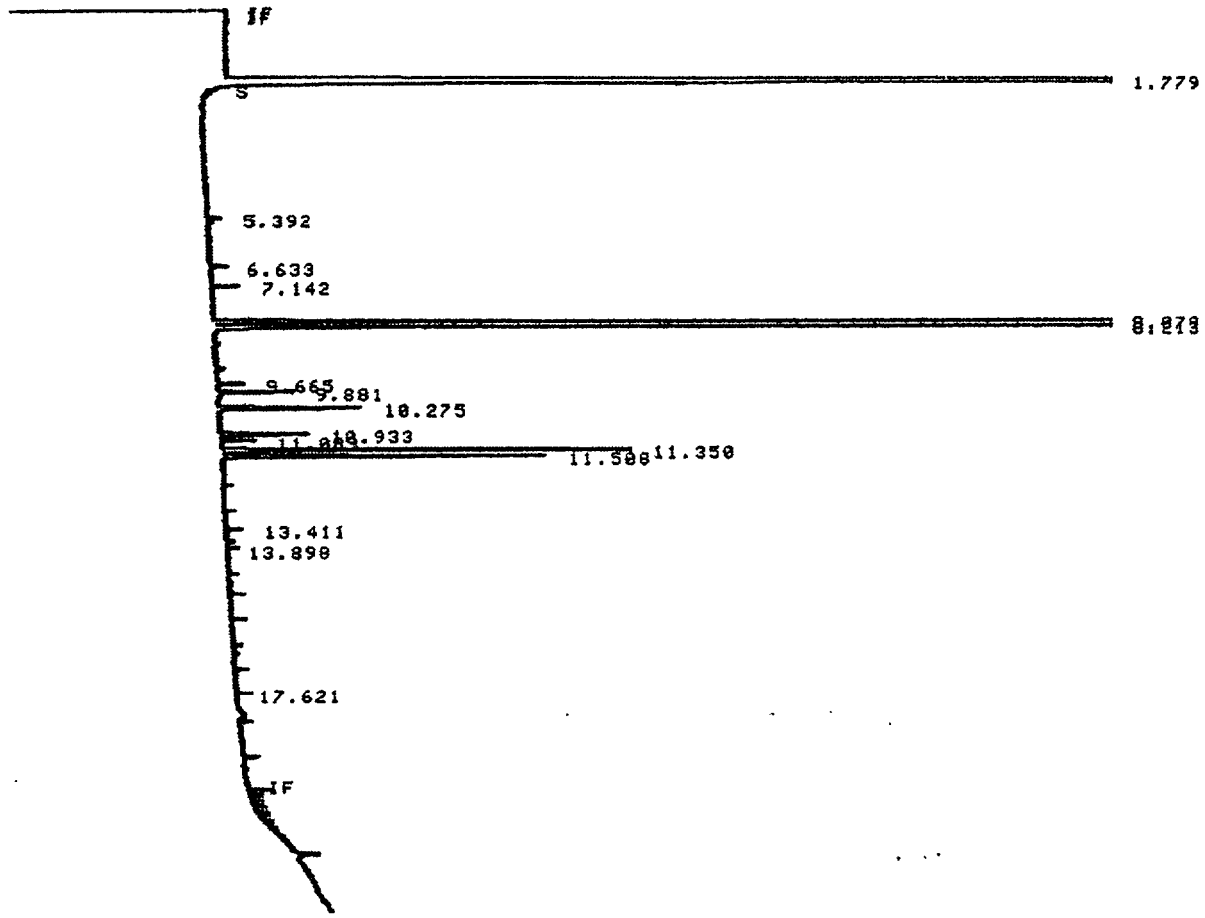


STOP

[redacted]

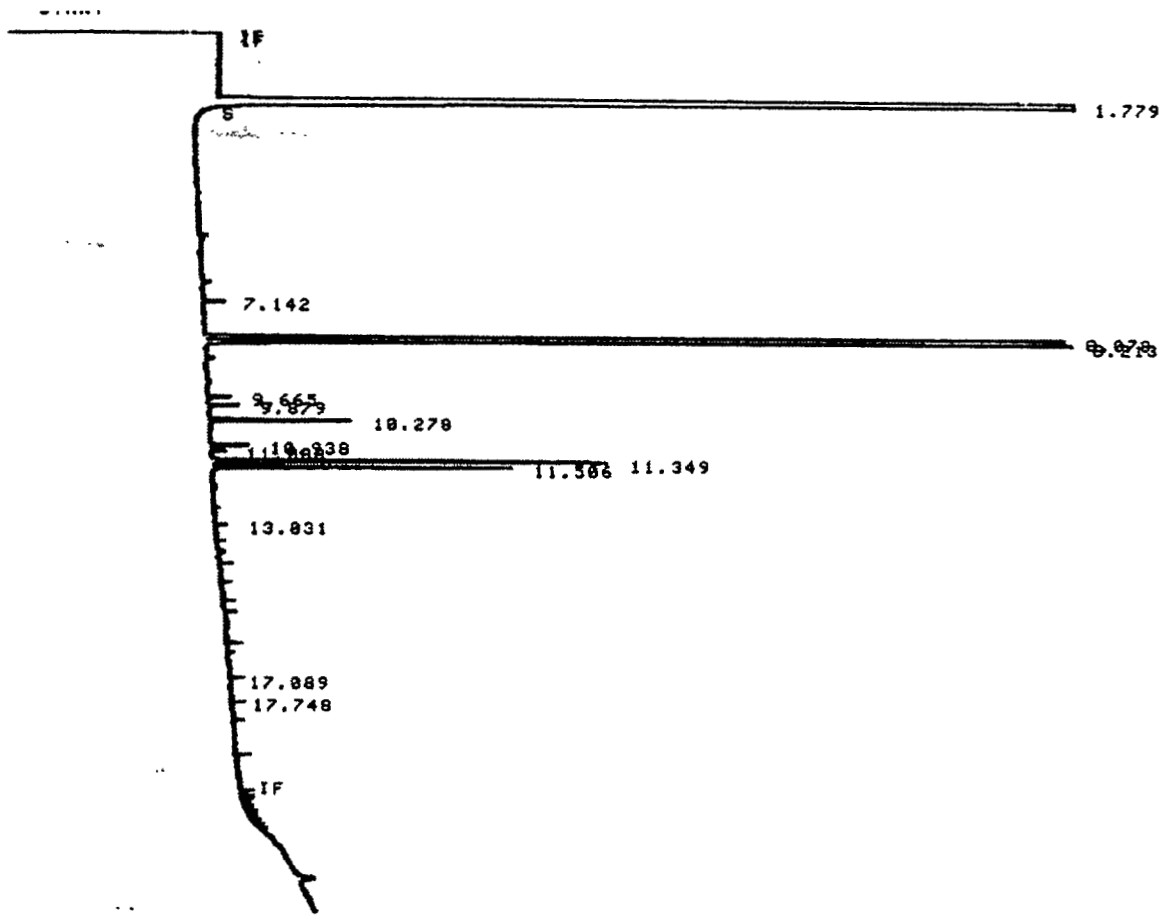
BOTTLE: 16 ID# 1492 WED 27-May-92 00:49:30

FILE DATA: F92526545

UN-ENZYME-BIO-SYS-5 (EBS-2 DM

RUN # 49 MAY 27, 1992 17:26:11

START



STOP

[redacted]

MIDI DOS SYSTEM 26-MAY-92 15:08:55

ID: 1491 UN-ENXYME-BIO-SYS-5 <EBS-1 DM Date of run: 27-MAY-92 16:55:40
Bottle: 15 SAMPLE [AEROBE]

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1.790	379861504	0.034	...	7.037	SOLVENT PEAK	...	< min rt	
6.648	688	0.043	0.990	13.618	14:0 ISO	0.44	ECL deviates -0.000	Reference -0.001
7.154	1160	0.044	0.979	13.999	14:0	0.74	ECL deviates -0.001	Reference -0.001
8.089	51696	0.044	0.964	14.622	15:0 ISO	32.39	ECL deviates 0.001	Reference 0.002
8.223	57016	0.044	0.962	14.712	15:0 ANTEISO	35.65	ECL deviates 0.001	Reference 0.001
9.677	1360	0.052	0.945	15.625	16:0 ISO	0.84	ECL deviates -0.001	Reference -0.001
9.890	3336	0.048	0.943	15.756	16:1 A	2.05	ECL deviates -0.001	
10.286	6264	0.048	0.939	15.999	16:0	3.83	ECL deviates -0.001	Reference -0.002
10.945	4144	0.050	0.935	16.386	17:1 ISO E	2.52	ECL deviates -0.001	
11.097	1672	0.051	0.934	16.475	Sum In Feature 5	1.02	ECL deviates -0.001	17:1 ISO I/ANTEI B
11.359	18872	0.049	0.932	16.629	17:0 ISO	11.44	ECL deviates 0.000	Reference -0.002
11.517	15016	0.049	0.931	16.722	17:0 ANTEISO	9.09	ECL deviates 0.000	Reference -0.002
******	1672	.....	.....	.....	SUMMED FEATURE 5	1.02	17:1 ISO I/ANTEI B	17:1 ANTEISO B/I I

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	ECL Deviation	Ref ECL Shift
379861504	161224	161224	100.00	153791	8	0.001	0.002

TSBA [Rev 3.50] Bacillus 0.0854
B. subtilis 0.0854
B. amyloliquefaciens ..... 0.731 (Bacillus subtilis group)

[redacted]

MIDI DOS SYSTEM 26-MAY-92 15:08:55

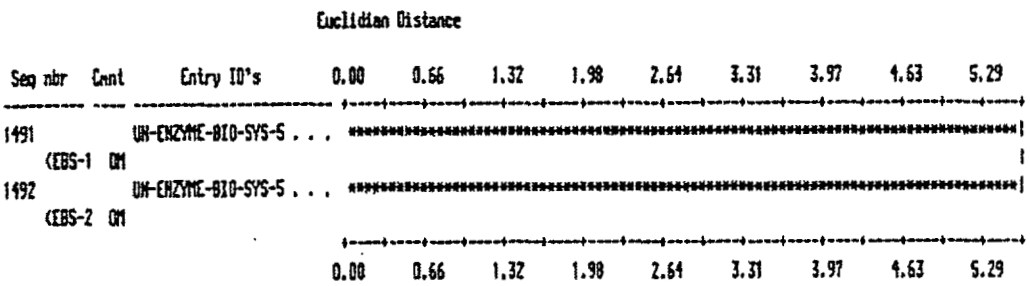
ID: 1492 UN-ENXYME-BIO-SYS-5 <EBS-2 DM Date of run: 27-MAY-92 17:26:11
Bottle: 16 SAMPLE [AEROBE]

RT	Area	Ar/Ht	Respon	ECL	Name	%	Comment 1	Comment 2
1.790	371466752	0.034	...	7.034	SOLVENT PEAK	...	< min rt	
7.154	928	0.045	0.979	13.999	14:0	0.76	ECL deviates - 0.001	Reference -0.001
8.088	36432	0.044	0.964	14.622	15:0 ISO	29.23	ECL deviates 0.001	Reference 0.001
8.223	43968	0.045	0.962	14.712	15:0 ANTEISO	35.20	ECL deviates 0.001	Reference 0.001
9.677	1048	0.050	0.945	15.626	16:0 ISO	0.82	ECL deviates - 0.000	Reference -0.001
9.889	1344	0.046	0.943	15.755	16:1 A	1.06	ECL deviates - 0.002	
10.286	6640	0.048	0.939	15.999	16:0	5.19	ECL deviates - 0.001	Reference -0.002
10.946	1880	0.050	0.935	16.387	17:1 ISO E	1.46	ECL deviates - 0.000	
11.360	19296	0.050	0.932	16.630	17:0 ISO	14.98	ECL deviates 0.001	Reference -0.001
11.516	14568	0.050	0.931	16.722	17:0 ANTEISO	11.30	ECL deviates - 0.000	Reference -0.003

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	ECL Deviation	Ref ECL Shift
371466752	126104	126104	100.00	120106	7	0.001	0.002

TSBA [Rev 3.50] Bacillus ..... 0.714 (Bacillus subtilis group)
B. subtilis ..... 0.688 (Bacillus subtilis group)

[redacted]



Samples link below 6 E.D., indicating same biotype or sub-species of B. subtilis

[redacted]

Appendix 3: Cloning, Sequencing and Expression of the *Bacillus naganoensis* Pullulanase Gene

The following is a summary report by ThermoGen, Inc. (Chicago Technology Park, 2201 West Campbell Park Drive, Chicago, IL 60612) describing the construction of *Bacillus subtilis* B1163/pEB301, which contains the *B. naganoensis* pullulanase gene.

We have successfully completed the transfer of the pullulanase gene from *Bacillus naganoensis* chromosome to *B. subtilis* strain B1-163. In keeping with project goals, we moved the gene to the Enzyme Bio-Systems ("EB") pCPC717 by essentially displacing the *alpha*-amylase gene contained in it. There were three complications we had to overcome. First, preliminary attempts at cloning the enzyme established that active expression of this gene in *Escherichia coli*, either on a plasmid or a phage, would not occur. We cloned inactive, overlapping gene fragments so that we could clone the entire gene DNA sequence into *B. subtilis*.

Second, we established that the *B. naganoensis* pullulanase gene promoter and leader caused instability in *B. subtilis*. We overcame that instability by using the *alpha*-amylase promoter and leader sequence existing in pCPC717. To take advantage of that sequence, we slightly modified the plasmid to introduce a useful restriction site just after the leader sequence. This modified plasmid we named pEB200.

Finally, we experienced extremely low transformation rates with strain B1-163. We first transformed an intermediate strain, B1-109, which is a high protease producing precursor of the target host. Transformation rates were poor in that strain as well, but we were successful with this avenue of first transforming strain B1-109, then strain B1-163.

The cloning is described below and in Figure 1. All DNA primer labels used below correspond to notes in ThermoGen laboratory records.

Derivation of Plasmid pEB301 that Contains the *Bacillus naganoensis* Pullulanase Gene

A. Modification of plasmid pCPC717.

000109

As stated above, we needed to place the mature pullulanase gene sequence into pCPC717, which contains a kanamycin resistance gene, just after the leader sequence of *alpha*-amylase. We could not identify a useful restriction site in that area so we made a single base pair change in pCPC717 to introduce a *Kpn*1 restriction site (Figure 1, Step 1). The change was to convert GGCACC to GGTACC at base pair 480. This base pair change causes no amino acid change. This slightly modified plasmid is

[redacted]

labeled pEB200 (Figure 1, Step 1).

B. Generation of pullulanase DNA probe for detecting clones.

We used the protein N-terminal amino acid sequence and the sequence of an internal, trypsin digest fragment to design DNA oligonucleotide probes.

1. **N-terminal** Asp-Gly-Asn-Thr-Thr-Asn-Ile-Val-Val-His-Tyr-Phe-Arg-Pro
   Primer D 5'-GA(T/C)-GGN-AA(T/C)-ACN-ACN-AA(T/C)-AT(A/T/C)-GTN-GTN-CA(T/C)-TA-3'
2. **Internal** Tyr-Asn-Val-Pro-Glu-Gly-Tyr-Gln
   Primer C 5'-TCC-TTC-AGG-GAC-GTT-GTA-3'

Polymerase chain reaction ("PCR") amplification of these oligonucleotides gave a 1.6kb fragment that we cloned into a pCR™II plasmid,[1] which contains a kanamycin resistance gene. Initial sequence data from this clone resulted in the design of two efficient primers for the isolation of the same probe.

3. **IND** 5'-ATG-TGG-CCG-GAG-AAC-GGT-GAT-GG-3'
4. **INC** 5'-GTT-GCG-AGG-GTC-ATA-ACC-CCA-AT-3'

C. Generation of lambda phages encoding pieces of the pullulanase gene.

Next, we made a DNA library of the *B. naganoensis* chromosomal DNA for use in screening with the 1.6kb DNA probe. We partially digested chromosomal DNA with *Sau*3A restriction enzyme; mixed the digest with the lambda-based, ZAP Express,™ *Bam*H1/CIAP-treated vector,[2] and joined with T4 DNA ligase. We constructed phage particles by encapsidation[3] *in vitro*. We infected *E. coli* XL1-Blue MRF',[4] grew them on LB agar plates, transferred the phage from plaques to HATF membranes,[5] and screened by hybridization to the 1.6kb, digoxigenin-labeled[6] probe.

We purified virus from plaques yielding positive screen results, prepared DNA from each of the positive phages, and determined the insert sequence. Figure 2 shows overlapping regions of inserts from two such phages, labeled as "Phage 7" and "Phage 10."

D. Cloning of the gene into a pCR™II plasmid.

We constructed the two oligonucleotides listed below using the known N-terminal and C-terminal protein sequence. The primers were designed so that the PCR product contains a *Kpn*1 site at the 5' end and a *Bam*H1 site at the 3' end.

5. **N-terminal** ***Kpn*1**
   #227 5'-GGG-<u>GGT-ACC</u>-GAT-GGG-AAC-ACC-ACA-AAC-ATC-G-3'
6. **C-terminal (after termination)**
   ***Bam*H1**
   PR-3 5'-<u>GGA-TCC</u>-TAA-GTT-CAT-TTA-GGT-CGA-TGA-AGG-3'

000110

Using them, we generated mature pullulanase gene DNA by PCR amplification directly from chromosomal DNA. We cloned the gel purified, PCR fragment into a pCR™II plasmid (Figure 1, Step 2), which has unique sites for *Kpn*1 and *Bam*H1, and transformed the ligation mixture into the *E. coli* K-12 derivative, INVαF'.[7] The resulting

[redacted]

chimera plasmid contains the pCR™II plasmid along with the mature pullulanase gene minus its promoter and leader sequences.

E. Cloning of the pullulanase gene into *Bacillus subtilis* strain B1-163

A large scale plasmid DNA preparation was made from the chimera clone containing the pullulanase gene and from the pEB200 plasmid. Digesting the plasmid DNA with *Kpn*1 and *Bam*H1 and ligation generated pEB301, a new plasmid containing the mature pullulanase gene in place of the *alpha*-amylase gene of pEB200 (Figure 1, Step 3). We transformed the ligation mixture into *B. subtilis* strain B1-109 and grew those cells on plates of double strength LB agar. The 1.5% agar plates contained 1% amylopectin and 40 µg/ml kanamycin. Positively transformed cells produced halos of clearing due to the pullulanase catalyzed hydrolysis of the amylopectin.

Finally, we isolated plasmid DNA from successfully transformed cells and introduced it into strain B1-163 using the same transformation and detection system described above.

N-terminal Sequence of the Recombinant Pullulanase Gene

The recombinant pullulanase protein was purified and the N-terminal sequence determined. The protein is processed at the same position as the *B. stearothermophilus alpha*-amylase leader. The new protein N-terminal is

Ala-Ala-Pro-Phe-Asn-Gly-Thr-Asp-Gly-Asn-...

The bold amino acids are the first seven amino acids of the *B. stearothermophilus alpha*-amylase. The pullulanase protein sequence follows.

To summarize, the final construct, pEB301, then contains the sequence for the *B. stearothermophilus alpha*-amylase promoter, leader and code for the first seven amino acids from pEB200. This is followed by the entire, mature *B. naganoensis* pullulanase gene from the chimeric plasmid. The remaining sequence is from pEB200.

[redacted]

Notes

[1] Invitrogen Corporation, 3985 B Sorrento Valley Blvd., San Diego, CA 92121.

[2] ZAP Express™ Predigested Vector Kit (Catalog #239212); Stratagene Cloning Systems, 11011 North Torrey Pines Road, La Jolla, CA 92037.

[3] Gigapack® III Gold-4 Packaging Extract (Catalog # 200201); Stratagene Cloning Systems.

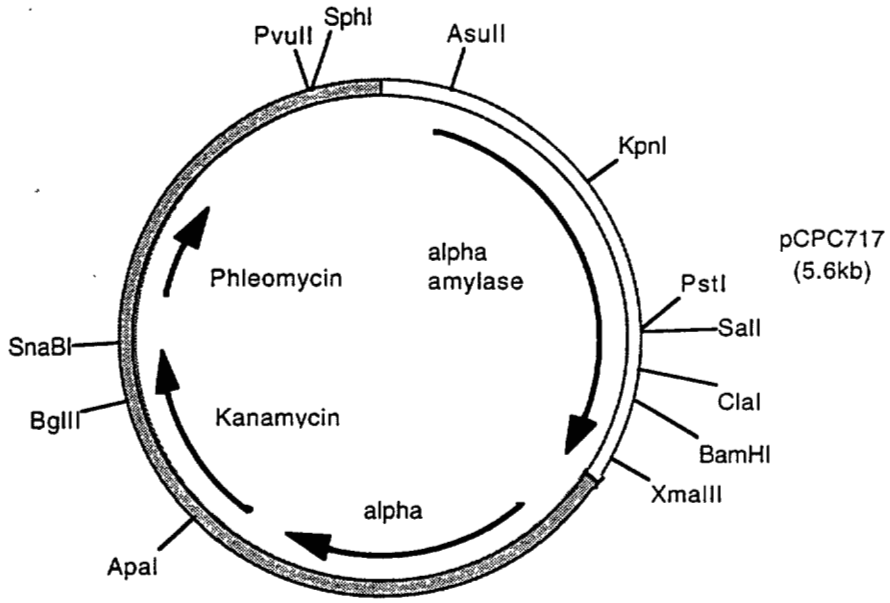
[4] ZAP Express™ Predigested Vector Kit; Stratagene Cloning Systems.

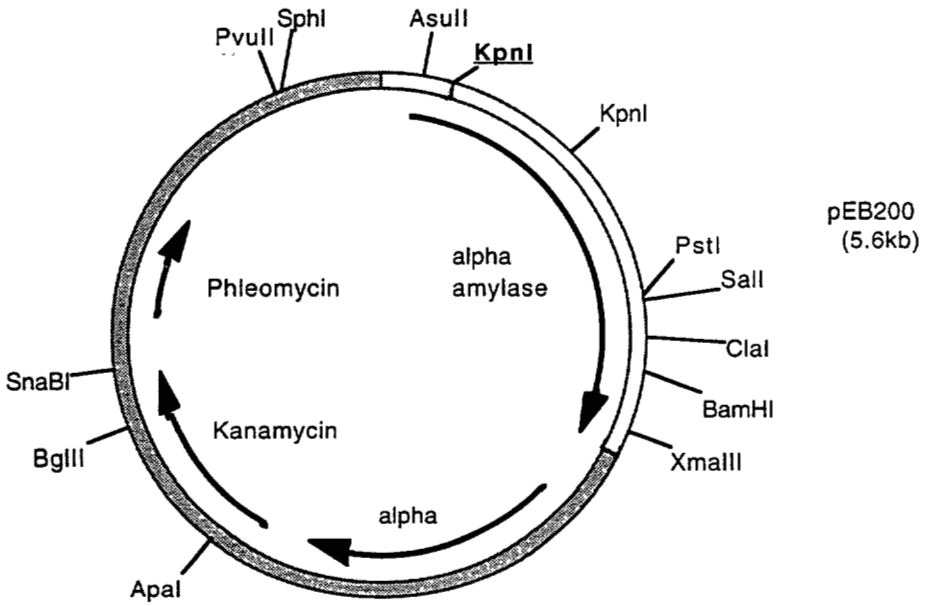
[5] Millipore Corporation, 80 Ashby Road, Bedford MA 01730.

[6] Genius 1, DNA Labeling and Detection Kit (Catalog # 1093 657); Boehringer Mannheim Corporation, 9115 Hague Road, PO Box 50414, Indianapolis, IN 46250-0414.

[7] Invitrogen Corporation.

[redacted]



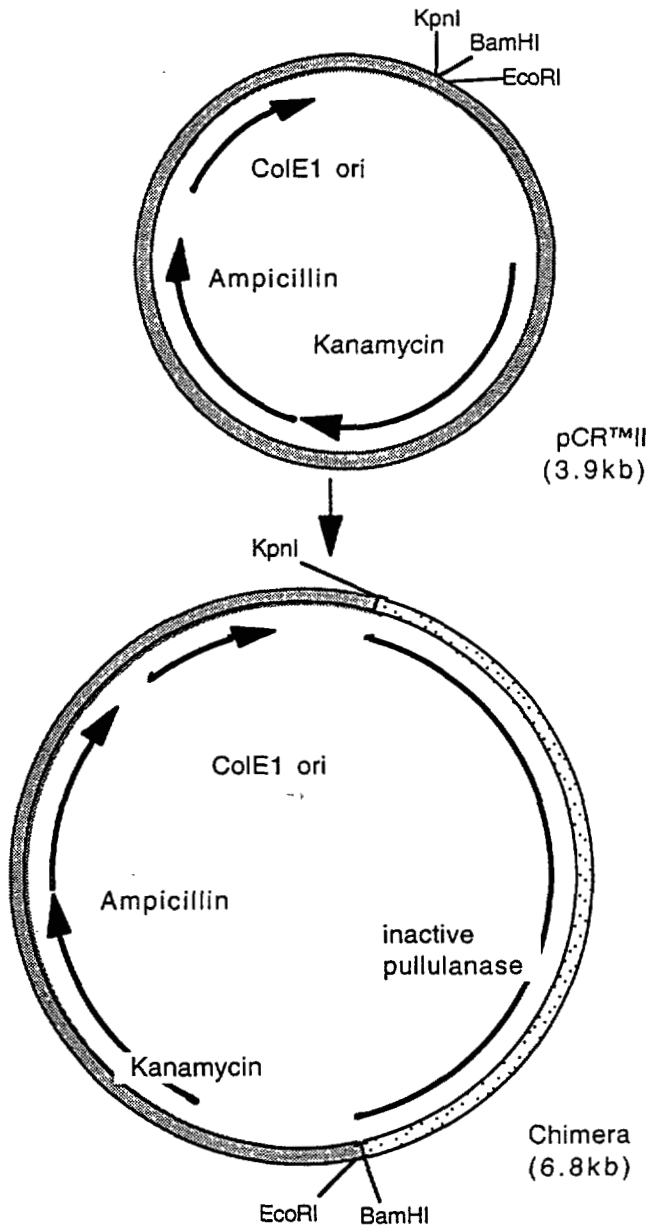


000113

Step 1. Prepare pCPC717 to Receive the Mature Pullulanase Gene.

Create pEB200, a modification of plasmid pCPC717, by introducing a *Kpn*1 restriction site. The change, converting GGCACC to GGTACC at base pair 480, causes no amino acid change.

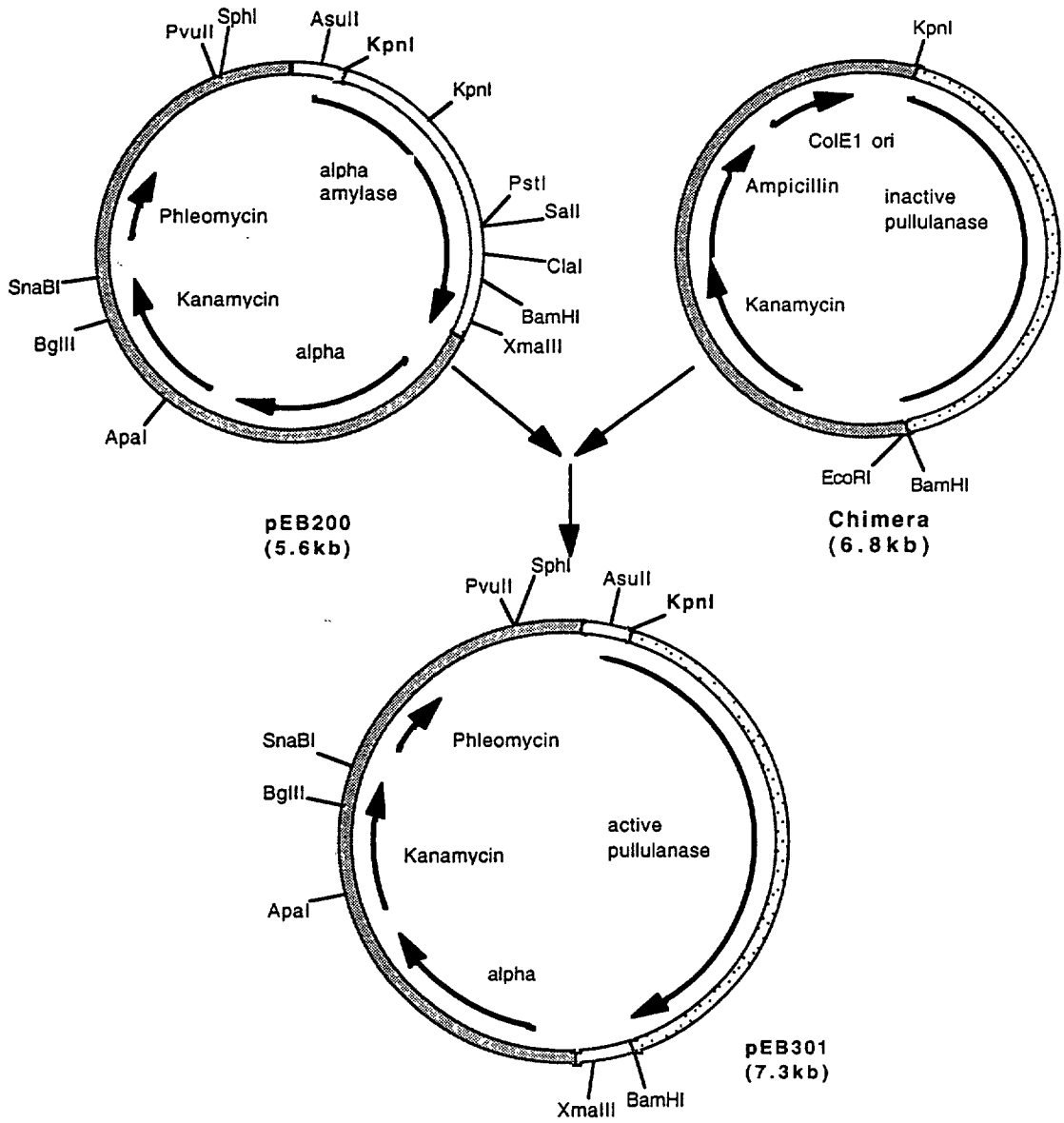
[redacted]



Step 2. Capture the Mature Pullulanase Gene from Chromosomal DNA.

Using primers to DNA sequences corresponding to the protein N-terminal and C-terminal regions and designed to contain a 5' *Kpn*1 site and a 3' *Bam*H1 site, we generated mature pullulanase gene DNA by PCR amplification directly from chromosomal DNA. We cloned it into a pCR™II plasmid, which has unique sites for *Kpn*1 and *Bam*H1, and transformed the ligation mixture into the *E. coli* K-12 derivative, INVαF'. The resulting chimera contains the pCR™II plasmid along with the mature pullulanase gene minus its promoter and leader sequences.

[redacted]



Step 3. Prepare the Production Plasmid, pEB301.

The chimeric plasmid containing the mature pullulanase gene and the pEB200 plasmid are digested with *Kpn*1 and *Bam*H1, purified, and ligated. The result is pEB301, which is composed of pEB200-derived sequence for *B. stearothermophilus alpha*-amylase promoter, leader and code for the first seven protein amino acids, and the chimera-derived mature *B. naganoensis* pullulanase gene.

[redacted]

Appendix 4: Transformability Testing of Pullulanase Product from *Bacillus subtilis* B1-163/pEB301

The following body of a memorandum from L. N. Allen, Director of Enzyme Development for ThermoGen, Inc. (Chicago Technology Park, 2201 West Campbell Park Drive, Chicago, IL 60612) contains evidence that the pullulanase product contains no transformable DNA.

000116

[redacted]

June 24, 1998

TO: W. M. Teague, Ph.D.

FROM: L. N. Allen, Ph.D.

Subject: Transformability Testing of Pullulanase Product from *Bacillus subtilis* B1-163/pEB301

Pullulanase product from *B. subtilis* B1-163/pEB301 was assessed for the presence of plasmid DNA in two ways:

A. Visual Assessment on Agarose Gels

Either 1 or 5 µl of pullulanase product (#941013; 2656 U/g) was run on agarose gels followed by staining with ethidium bromide to visualize DNA. No DNA or RNA was visible in lanes run with pullulanase product.

B. Transformability of *B. subtilis* Strain B1-109 with Pullulanase Product

The transformability of competent *B. subtilis* B1-109 cells by the pullulanase product was tested using the transformation protocol of Saunders *et al.* [1]

The data follow:

Sample	Kanamycin Resistance (transformants/ml)
Pullulanase [a]	0
Plasmid pEB301	4×10^3
Plasmid pUB110	4.5×10^4
No DNA	0 [b]

Legend
a. Enzyme Bio-Systems Ltd. Production Lot 941013 (50 µl)
b. 5.6×10^8 viable cells /ml

Conclusion

No *B. subtilis* B1-163/pEB301 DNA was found in pullulanase product by visualization on agarose gels or by transformation using competent *B. subtilis* cells.

[1] Transformation of Bacteria by Plasmid DNA. 1984. Saunders, J. R., A. Docherty, and G. O. Humphries in: Methods in Microbiology, Vol. 17, pp. 61-95.

[redacted]

Appendix 5: TOXICOLOGICAL SUMMARY - 14-Day Acute Oral Toxicity Study of *Bacillus naganoensis* Pullulanase Enzyme in Rats

The toxicity study, executed using *Bacillus naganoensis* pullulanase, was done by Hazleton Wisconsin, Inc. (3301 Kinsman Boulevard, Madison, Wisconsin 53704 USA). A summary of report HWI 6388-102 follows.

(a) Purpose/objective of the experiment

The objective of this study was to access the acute oral toxicity produced when *Bacillus naganoensis* pullulanase was administered by the oral route (gavage) to rats.

(b) Identification of the substance administered

The test material, pullulanase enzyme derived from *B. naganoensis*, Batch No. D39-001-2, was used for this study.

(c) Designation of animal species and strain employed

Young adult albino rats of the Crl:CD®(SD)BR strain from Charles River Laboratories, Inc., Portage, Michigan

(d) Number of animals in test and control groups

Twenty animals in the test group and the control group

(e) Sex of animals in test and control groups

Ten male and 10 female rats were in the test group and the control group

(f) Dose levels of treatment

For Group 2, the test material was administered as received to achieve a dose level of 5,000 mg/kg of body weight. The vehicle control animals (Group 1) were treated with deionized water at the same dose volume as the test group. An individual dose of the test material mixture or deionized water was calculated for each representative animal based on its fasted body weight and administered as a single gavage dose.

000118

(g) Route of administration

Historically, the oral route has been the route of choice for administering a known amount of test material for acute oral toxicity determination. Furthermore, the intended route of administration in humans is oral.

[redacted]

(h) Duration of treatment

The in-life phase of the study lasted 14 days.

(i) Biological parameters examined

Clinical Observations

Clinical observations and mortality checks were conducted at 1, 2.5, and 4 hours after test material administration and daily thereafter for 14 days. Twice a day mortality checks (morning and afternoon) were conducted daily thereafter for 14 days. Qualitative food consumption and the general appearance of the feces were also noted once daily. Body weights were determined on Day -1 (for randomization purposes), before test material administration (Day 0), at Day 7, and at termination of the in-life phase (Day 14).

Pathology

At termination of the in-life phase, all animals were euthanized, subjected to an abbreviated necropsy examination, and any abnormalities were recorded. After necropsy, the animals were discarded and no tissues were saved.

(j) Effects observed

No mortality was observed during the study. All animals exhibited body weight gains throughout the study.

All animals in Group 1 (vehicle control group) appeared normal throughout the study with the exception of three animals which exhibited small feces on various days during the study. One of these animals also exhibited below normal food consumption on Day 1.

Small feces and/or soft stool were observed in 8 test animals (Group 2) on various days during the study. Light-colored stool was also exhibited by one test animal on Day 6. In addition, below normal food consumption was observed in three test group animals on various days during the study. These are not unusual findings in rats when given a large volume of a highly concentrated test mixture. All remaining test group animals appeared normal throughout the study.

There were no test material related lesions observed at the gross necropsy examinations at termination.

(k) Conclusions

The acute oral toxicity of pullulanase enzyme derived from *Bacillus naganoensis* was evaluated in male and female rats when administered as a single gavage dose at a level of 5,000 mg/kg of body weight. The estimated oral LD_{50} value for this enzyme when given to male and female rats was determined to be greater than 5,000 mg/kg.

[redacted]

Appendix 6: Tabular Representation of Genotoxicity Data for Pullulanase Derived from *Bacillus naganoensis*

Test System[1]	Test Object	Concentration of Test Material	Result
Ames Test [2]	*S. typhimurium* TA98,TA100, TA1535, TA1537, TA1538	333-5,000 µg/plate	negative
Micronuclei Induction Test [3]	Bone marrow polychromatic erythrocytes of CD-1(ICR) mice	1,250-5,000 mg/kg	negative
Forward Mutation Assay [4]	Thymidine kinase (TK) locus in mouse lymphoma L5178Y cell line	500-5,000 µg/ml	negative

1. All tests were done by Corning Hazleton Inc. (9200 Leesburg Pike, Vienna, Virginia 22182 USA) on *B. naganoensis* pullulanase, Batch D39-001-2.
2. The Ames Test, or Salmonella / Mammalian -- Microsome Reverse Mutation Assay, was done both with and without rat liver S9 mix (report CHV 16516-0-401).
3. The result of the test, also called the *In Vivo* Mouse Micronucleus Assay, is contained in Hazleton report 16516-0-455.
4. L5178Y Tk +/- Mouse Lymphoma Forward Mutation Assay was done both with and without rat liver S9 mix (Hazleton report 16516-0-431).

[redacted]

Appendix 7: TOXICOLOGICAL SUMMARY - 4-Week Dietary Toxicity Study with *Bacillus naganoensis* Pullulanase in Rats

The toxicity study was done by Corning Hazleton Inc. (3301 Kinsman Boulevard, Madison, Wisconsin 53704 USA). The summary of report HWI 6388-101 follows.

(a) Purpose/objective of the experiment

The purpose of this study was to evaluate the toxicity including neurobehavioral toxicity of *Bacillus naganoensis* pullulanase when administered daily in the diet to rats for at least 4 weeks.

(b) Identification of the substance administered

The test material, pullulanase enzyme derived from *B. naganoensis*, Batch D39-002, was used for this study.

(c) Designation of animal species and strain employed

Crl:CD®(SD)BR VAF/Plus® rats obtained from the Portage, Michigan, facility of Charles River Laboratories, Inc.

(d) Number and sex of animals in test and control groups/dosage levels of treatment

Group	Dosage Level (%)[a]	Number of Animals male	female
1 (Control)	-	10	10
2 (Low)	0.75	10	10
3 (High)	1.5	10	10
Baseline	-	5	5[b]

a. The control group received the basal diet only.
b. Five animals per sex were used for baseline clinical pathology, then sacrificed and discarded.

(e) Route of administration

Dietary admixture was used because the intended route of administration in humans is oral.

(f) Duration of treatment

The dose preparations were administered *ad libitum* for at least 28 consecutive days.

(g) Biological parameters examined

Clinical Observations

The animals were observed twice daily (a.m. and p.m.) for mortality and moribundity. Signs of poor health or abnormal behavior were recorded as they were observed. At least once each week, each animal was observed by removing the animal from its cage; abnormal findings or an indication of normal was recorded.

Body Weights and Food Consumption

Body weights were recorded on the first day of treatment and weekly thereafter. Individual food consumption data were collected weekly.

Ophthalmology

Ophthalmic examinations were done by a veterinary ophthalmologist before initiation of the treatment (all animals) and during Week 4 (control and high-dose animals only). The pupils were dilated with 0.5% Mydriacyl® and the eyes were examined with an indirect ophthalmoscope.

Neurotoxicity Tests

The neurobehavioral tests were done on all animals once during Week 4. Technicians doing the Functional Observational Battery (FOB) were unaware of the dose level of each animal.

Functional Observational Battery

The FOB consisted of a series of observations made of the animals in the cage, during handling, in an open arena, and during manipulations to assess reflexes and physiological parameters. Before removal from the home cage, the animals were evaluated for position, activity, body tone, tremor, convulsions, vocalization, palpebral closure, exophthalmus, excessive lacrimation, excessive salivation, respiration, appearance of fur, piloerection, and writhing. Each animal was placed into an open field arena; the latency (time in seconds) to the first step was recorded, and the animals were observed for position, gait abnormalities (type and severity), activity, stereotypy, body tone, tremor, convulsions, and other unusual behavior. In addition, the number of grooms, rears, fecal boli, and urine pools were recorded.

Each animal's response to being approached with an object (penlight) and the response to touch with the penlight were evaluated; catalepsy was evaluated by recording whether the condition was present or absent. Animals were evaluated for pupillary status, pupillary response, and corneal response to touch. Startle response, air drop righting reflex, analgesic reflex, hindlimb and forelimb grip strength, hindlimb foot splay, and body temperature were also

[redacted]

assessed, and a body weight was taken.

Clinical Pathology

Blood and urine samples were collected from all animal before scheduled sacrifice. Animals were fasted overnight and urine was collected for approximately 16 hours before the blood sampling. Water was provided *ad libitum*. Blood was collected from a jugular vein. Potassium EDTA was used as the anticoagulant for hematology tests. Animals were bled in random order. The following were evaluated if sufficient sample was available:

Hematology
- red blood cell (erythrocyte) count
- hemoglobin
- hematocrit
- mean corpuscular volume
- mean corpuscular hemoglobin
- mean corpuscular hemoglobin concentration
- platelet count
- white blood cell (leukocyte) count
- blood cell morphology
- differential blood cell count
  - nucleated red blood cell count
  - corrected white blood cell count
  - segmented neutrophil count
  - band neutrophil count
  - lymphocyte count
  - monocyte count
  - eosinophil count
  - basophil count

Reticulocyte count smears were made and held for possible future examination.

Clinical Chemistry
- glucose
- urea nitrogen
- creatinine
- total protein
- albumin
- globulin
- cholesterol
- total bilirubin
- alanine aminotransferase
- alkaline phosphatase
- gamma-glutamyl transferase
- aspartate aminotransferase
- calcium
- inorganic phosphorus
- sodium

[redacted]

potassium
chloride

Urinalysis
specific gravity
pH
protein
glucose
ketones
bilirubin
blood
urobilinogen
volume
microscopic examination of sediment
appearance

Anatomical Pathology

After 4 weeks of treatment, animals were fasted overnight, anesthetized with sodium pentobarbital, weighed, bled for clinical pathology tests, exsanguinated, and necropsied. Animals were necropsied in random order. The necropsy included a macroscopic examination of the external surface of the body; all orifices; the cranial cavity; the brain and spinal cord; the nasal cavity and paranasal sinuses; the thoracic, abdominal, and pelvic cavities and viscera. At the scheduled sacrifice, the following organs (when present) were weighed; paired organs were weighed separately:

adrenal (2)
brain
kidney (2)
liver
ovary (2)
spleen
testis (2)
thyroid (2) with parathyroid
thymus

Organ-to-body weight percentages and organ-to-brain weight ratios were calculated. The following tissues (when present) or representative samples were collected and preserved in 10% phosphate-buffered formalin:

adrenal (2)
auditory sebaceous glands
aorta
brain
cecum
cervix
colon
duodenum

[redacted]

epididymis (2)
esophagus
eye (2)
femur with bone marrow (articular surface of distal end)
Harderian gland
heart
ileum
jejunum
kidney (2)
lesions
liver
lung with mainstem bronchi
lymph node (mesenteric and submaxillary)
mammary gland (females only)
nasal turbinates
ovary (2)
pancreas
pituitary
prostate
rectum
salivary gland [submaxillary (2)]
sciatic nerve
seminal vesicles
skeletal muscle (thigh)
skin
spinal cord (cervical, mid-thoracic, and lumbar)
spleen
sternum with bone marrow
stomach
testis (2)
thymus
thyroid (2) with parathyroid
trachea
urinary bladder
uterus
vagina

Tissues (as appropriate) were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically from each animal. Bone marrow smears from the femur of each animal at the scheduled sacrifice were prepared, stained with Wright's stain, and retained for possible examination.

(h) Effects observed

One female given 0.75% was found dead during Week 5; this animal had no clinical findings before its death. All other animals survived until scheduled sacrifice. There were no test material-related antemortem observations.

[redacted]

There were no statistical differences in mean body weights. Mean cumulative body weight gain was 7% to 10% lower throughout the study for males given 1.5% test material compared with that of the control males and statistically lower beginning at Week 2 and continuing throughout the study. The differences in the mean cumulative body weight gains are considered to be due to decreased food consumption and are not considered adverse. Though not significantly different, the mean cumulative body weight gain for males given 0.75% was also slightly less than control males. There were no other statistical differences in mean cumulative body weight gains.

Food consumption was significantly lower (6%) at Week 4 for males given 0.75% diet and 7% to 9% lower throughout the study for males given 1.50% diet. These small decreases in food consumption in males given the 1.50% diet are considered to be due to decreased palatability of the *B. naganoensis* pullulanase diet and are not considered adverse. Food consumption for the treated females was similar to that of control females throughout the study.

The amount of test material consumed by the animals on a mg/kg of body weight/day basis range was 591.8 to 896.7 and 1,157.5 to 1,878.2 for animals given 0.75% and 1.50%, respectively.

Animals selected for the study had no ophthalmic lesions at the prestudy examination. No test material-related ophthalmic observations were noted at the Day 28 examination.

Cageside observations noted for animals during the FOB tests at Week 4 were unremarkable. The behavior of animals placed in the open field was also relatively unremarkable. Reflex and physiological parameters were similar among all groups. There were no statistical differences in the number of grooms, fecal boli, urine pools, or rears of the animals in open field. There were no significant differences in mean analgesic reflex, grip strength, hindlimb foot splay, or body temperature across the group.

Administration of pullulanase derived from *B. subtilis* B1-163/pEB301 had no effects on clinical pathology or anatomical pathology results. The death of one female was attributed to urolithiasis and pyelonephritis; this was not considered test material-related.

[redacted]

(i) Conclusions

Based on the results of this study, dietary administration of *B. naganoensis* pullulanase derived from *B. naganoensis* to male and female Crl:CD®(SD)BR VAF/Plus® rats for at least 4 weeks resulted in no adverse systemic effects. Lower mean cumulative body weights and lower food consumption were noted only in males given the 1.50% pullulanase diet and were small in magnitude (7% to 10%) during one week. These data suggest that the 1.50% pullulanase diet was less palatable to male rats. Overall mean body weights were similar across all groups and there were no adverse clinical findings in the animals given the 0.75% or the 1.50% diet. The pullulanase diet had no effect on the neurobehavioral, clinical pathology, or anatomical pathology results.

The no-observable-adverse-effect level of *B. naganoensis* pullulanase in rats is considered to be 0.75% for male rats and 1.50% for female rats.

[redacted]

Appendix 8: TOXICOLOGICAL SUMMARY - 14-Day Acute Oral Toxicity Study of *Bacillus naganoensis* Pullulanase from *Bacillus. subtilis* B1-163/pEB301 in Rats

The toxicity study, executed using pullulanase from *Bacillus subtilis* B1-163/pEB301, was done by Covance Laboratories, Inc. (PO Box 7545, Madison, WI 53707-7545, USA). A summary of Covance report 6388-103 follows.

(a) Purpose/objective of the experiment

The objective of this study was to access the acute oral toxicity produced when *Bacillus naganoensis* pullulanase derived from *Bacillus subtilis* B1-163/pEB301 was administered by the oral route (gavage) to rats.

(b) Identification of the substance administered

The test material, pullulanase enzyme derived from *B. subtilis* B1-163/pEB301, Batch 003, was used for this study.

(c) Designation of animal species and strain employed

Young adult albino rats of the Crl:CD®(SD)BR strain from Charles River Laboratories, Inc., Raleigh, North Carolina

(d) Number of animals in test and control groups

Twenty animals in the test group and the control group

(e) Sex of animals in test and control groups

Ten male and 10 female rats were in the test group and the control group

(f) Dose levels of treatment

For Group 2, the test material was mixed with deionized water to a concentration of 0.5 g/ml then administered to achieve a dose level of 5,000 mg/kg of body weight. The vehicle control animals (Group 1) were treated with deionized water. An individual dose of the test material mixture or deionized water was calculated for each representative animal based on its fasted body weight and administered as a single gavage dose at a volume of 10 ml/kg of body weight.

(g) Route of administration

Historically, the oral route has been the route of choice for administering a known amount of test material for acute oral toxicity determination. Furthermore, the intended route of administration in humans is oral.

(h) Duration of treatment

The in-life phase of the study lasted 14 days.

(i) Biological parameters examined

Clinical Observations

Clinical observations and mortality checks were conducted at 1, 2.5, and 4 hours after test material administration and daily thereafter for 14 days. Mortality checks were conducted twice a day (morning and afternoon) for 13 days after test material administration and again the morning of Day 14. Qualitative food consumption and the general appearance of the feces were also noted once daily. Body weights were determined before test material administration (Day 0), at Day 7, and at termination of the in-life phase (Day 14).

Pathology

At termination of the in-life phase, all animals were euthanized, subjected to an abbreviated necropsy examination, and any abnormalities were recorded. After necropsy, the animals were discarded and no tissues were saved.

(j) Effects observed

No mortality was observed during the study. All animals exhibited body weight gains throughout the study with the exception of two females in Group 1 (vehicle control group) and one female in Group 2 (test group) which exhibited a weight loss of 3 to 19 g during the second week.

All animals in Group 1 (vehicle control group) appeared normal throughout the study with the exception of one female which exhibited a red-stained face on Day 1.

Nonformed and/or small feces were observed in 13 test animals (Group 2) on various days during the first four days of the study. In addition, one female in Group 2 exhibited a red-stained face and another female was observed to have below normal food consumption on the day of dosing. These are not unusual findings in rats when given a large volume of a highly concentrated test mixture. All remaining test group animals appeared normal throughout the study.

There were no test material related lesions observed at the gross necropsy examinations at termination.

(k) Conclusions

The acute oral toxicity of pullulanase enzyme derived from *B. subtilis* B1-163/pEB301 was evaluated in male and female rats when administered as a single gavage dose at a level of 5,000 mg/kg of body weight. The estimated oral LD_{50} value for this enzyme when given to male and female rats was determined to be greater than 5,000 mg/kg.

[redacted]

Appendix 9: TOXICOLOGICAL SUMMARY - 4-Week Dietary Toxicity Study with *Bacillus naganoensis* Pullulanase from *Bacillus subtilis* B1-163/pEB301 in Rats

The toxicity study, executed using pullulanase from *Bacillus subtilis* B1-163/pEB301, was done by Covance Laboratories, Inc. (PO Box 7545, Madison, WI 53707-7545, USA). The summary of report HWI 6388-101 follows.

(a) Purpose/objective of the experiment

The purpose of this study was to evaluate the toxicity of *Bacillus naganoensis* pullulanase derived from *Bacillus subtilis* B1-163/pEB301 when administered daily in the diet to rats for at least 4 weeks.

(b) Identification of the substance administered

The test material, pullulanase enzyme derived from *B. subtilis* B1-163/pEB301, Lot No. 003, was used for this study.

(c) Designation of animal species and strain employed

Crl:CD®(SD)BR VAF/Plus® rats obtained from the Portage, Michigan, facility of Charles River Laboratories, Inc.

(d) Number and sex of animals in test and control groups/dosage levels of treatment

Group	Dosage Level (%)	Number of Animals male	female
1 (Control) [a]	-	10	10
2 (Low)	0.625	10	10
3 (High)	1.25	10	10

a. The control group received the basal diet only.

(e) Route of administration

Dietary admixture was used because the intended route of administration in humans is oral.

(f) Duration of treatment

The dose preparations were administered *ad libitum* for at least 28 consecutive days.

(g) Biological parameters examined

[redacted]

Clinical Observations

The animals were observed twice daily (a.m. and p.m.) for mortality and moribundity. Each animal was observed (cage was opened) daily for the first 2 weeks (except on days when daily observations were done); abnormal findings were recorded. Once weekly, each animal was observed by removing the animal from its cage; abnormal findings or an indication of normal was recorded.

Body Weights and Food Consumption

Body weights were recorded at least once prior to treatment, on the first day of treatment, and weekly thereafter. Food consumption data were collected weekly.

Ophthalmology

Ophthalmic examinations were done by a veterinary ophthalmologist before initiation of the treatment and on Day 28. The pupils were dilated with 0.5% Mydriacyl® and the eyes were examined with an indirect ophthalmoscope.

Clinical Pathology

Blood samples were collected from each animal during Week 5. During Week 5 urine samples were collected from each animal with the exception of Animals Nos. C85831, C85834, C85837, (Group 1 males); Animal No. C85847, (Group 2 male); Animal No. C85859, (Group 3 male); and Animal No. C85883, (Group 3 female), which were necropsied prior to urine collection at the terminal necropsy. The urine samples were collected for approximately 16 hours. Water was provided *ad libitum*. Animals were fasted overnight, and blood was collected from a jugular vein. Potassium EDTA was used as the anticoagulant for hematology tests. Animals were bled in random order. The following were evaluated:

Hematology
- red blood cell (erythrocyte) count
- hemoglobin
- hematocrit
- mean corpuscular volume
- mean corpuscular hemoglobin
- mean corpuscular hemoglobin concentration
- platelet count
- white blood cell (leukocyte) count
- differential blood cell count
  - segmented neutrophil count
  - lymphocyte count
  - monocyte count
  - eosinophil count
  - basophil count
- blood cell morphology

Reticulocyte count smears were made and held for possible future examination.

Clinical Chemistry
glucose
urea nitrogen
creatinine
total protein
albumin
globulin
cholesterol
total bilirubin
alanine aminotransferase
alkaline phosphatase
gamma-glutamyl transferase
aspartate aminotransferase
calcium
inorganic phosphorus
sodium
potassium
chloride

Urinalysis
appearance
specific gravity
pH
protein
glucose
ketones
bilirubin
blood
urobilinogen
volume
microscopic examination of sediment

Anatomical Pathology

At the scheduled sacrifice, the following organs (when present) were weighed; paired organs were weighed separately:

adrenal (2)
brain
kidney (2)
liver
ovary (2)
spleen
testis (2)
thyroid (2) with parathyroid
thymus

[redacted]

Organ-to-body weight percentages and organ-to-brain weight ratios were calculated. The following tissues (when present) or representative samples were collected and preserved in 10% neutral-buffered formalin:

adrenal (2)
auditory sebaceous glands
aorta
brain
cecum
cervix
colon
duodenum
epididymis (2)
esophagus
eye (2)
femur with bone marrow (articular surface of distal end)
Harderian gland
heart
ileum
jejunum
kidney (2)
lesions
liver
lung with mainstem bronchi
lymph node (mesenteric and mandibular)
mammary gland (females only)
nasal turbinates
ovary (2)
pancreas
pituitary
prostate
rectum
salivary gland [mandibular (2)]
sciatic nerve
seminal vesicles
skeletal muscle (thigh)
skin
spinal cord (cervical, mid-thoracic, and lumbar)
spleen
sternum with bone marrow
stomach
testis (2)
thymus
thyroid (2) with parathyroid
trachea
urinary bladder

[redacted]

uterus
vagina

Tissues (as appropriate) were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically from each animal in the control and high-dose groups. Bone marrow smears from the femur of each animal at the scheduled sacrifice were prepared, stained with Wright's stain, and retained for possible examination.

(h) Effects observed

There were no statistical differences in mean body weights. Mean body weight changes were statistically higher between Weeks 4 and 5 for males given 1.25% pullulanase; however, this is not considered to be toxicologically important.

There were no statistical differences in mean food consumption between the groups.

Animals were fed diets containing 0.625% and 1.25% pullulanase enzyme product. The mean amount of test product consumed by the animals in these groups ranged from 466 to 659 mg/kg/day and from 914 to 1,313 mg/kg/day for males, respectively, and from 540 to 664 mg/kg/day and from 995 to 1226 mg/kg/day for females, respectively.

Animals selected for the study had no ophthalmic lesions at the prestudy examination. No test material-related ophthalmic observations were noted at the Day 28 examination. Administration of pullulanase derived from *B. subtilis* B1-163/pEB301 had no effects on clinical pathology or anatomical pathology results.

(i) Conclusions

Based on the results of this study, dietary administration of *B. naganoensis* pullulanase derived from *B. subtilis* B1-163/pEB301 to male and female Crl:CD®(SD)BR VAF/Plus® rats for at least 4 weeks resulted in no effects at levels up to and including 1.25% dietary.

[redacted]

SUBMISSION END

000135