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1001 G STREET, N. W.
SUITE 500 WEST
WASHINGTON, D.C. 20001
TEL. 202.434.4100
FAX 202.434.4646
WWW.KHLAW.COM

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WRITER'S DIRECT ACCESS

John B. Dubeck
(202) 434-4125
Dubeck@khlaw.com

March 24, 2003

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Hand Delivered

Dr. Alan Rulis
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, Maryland 20740-3835

APR - 1 2003

Re: GRAS Notification for BD5088 alpha-amylase enzyme preparation, derived from *Pseudomonas flourescens* Biovar I, expressing a gene encoding an optimized *Thermococcales* alpha-amylase

Dear Dr. Rulis:

Pursuant to proposed 21 C.F.R. § 170.36(c) and on behalf of our client, Innovase LLC, we hereby notify the agency of our determination on the basis of scientific procedures that the above referenced alpha-amylase enzyme preparation is generally recognized as safe (GRAS) when used as a processing aid to hydrolyze edible starch in the production of starch hydrolysis products such as high fructose corn syrup, other sweeteners, and ethanol. As with all GRAS substances, the enzyme preparation when used in this application is exempt from the premarket clearance requirement applicable to food additives under section 409 of the Food, Drug, and Cosmetic Act.

We trust you will find the enclosed notification acceptable. Should any questions arise during the review process, please do not hesitate to contact us, preferably by telephone, so that we may respond as quickly as possible.

Very truly yours,

(b)(6)

John B. Dubeck

Enclosure (GRAS Notification in triplicate)

cc: David Hanselman

INNOVASE®

Alpha-Amylase Derived from *Pseudomonas fluorescens* Biovar I Expressing a Gene Encoding an Optimized *Thermococcales* Alpha-Amylase

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A Joint Venture of The Dow Chemical Company and Diversa Corporation

Innovase LLC
5501 Oberlin Drive
San Diego, CA 92121 USA

Tel. (858) 352-4454
Fax (858) 352-4560

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Introduction

The α-amylase enzyme preparation that is the subject of this Notification, referred to as α-amylase BD5088, is intended for use in the hydrolysis of edible starch to produce starch hydrolysis products, including glucose syrup, high fructose corn syrup (HFCS), and crystalline glucose (dextrose), and distilled ethanol for food and beverage use. Alpha-amylase hydrolyzes the α-1-4 linkages in starch during wet milling. Treatment of starch with α-amylase may be followed by treatment with other suitable enzymes such as glucoamylase, pullulanase, and glucose isomerase.

The BD5088 α-amylase enzyme is derived from a genetically modified strain of *Pseudomonas fluorescens* Biovar I.

As discussed in greater detail in Section III.9.d below, residues of the BD5088 α-amylase enzyme in processed sweeteners are not detectable at a sensitivity of 2.4 ppb. The corresponding dietary concentration for BD5088 total organic solids (TOS) is less than 1 ppb.

The safety of the BD5088 α-amylase enzyme preparation when used as described is supported by a series of safety studies including a 90-day oral toxicity study in rats, *in vitro* and *in vivo* genotoxicity studies on the enzyme preparation, and a pathogenicity study on the host microorganism. The NOAEL derived from the 90-day rat feeding study is approximately 44,500,000 times higher than reasonably anticipated *per capita* exposure to the TOS present in BD5088 α-amylase.

A GRAS Panel of highly qualified experts has reviewed the safety data upon which this GRAS Notification relies and has deemed the BD5088 α-amylase enzyme preparation to be safe when used as described.

I) Administrative

I. Administrative Information

1. Claim Regarding GRAS Status

Innovase LLC hereby notifies the Agency of its determination that alpha-amylase enzyme preparation, derived from *Pseudomonas fluorescens* Biovar I, expressing a gene encoding an optimized *Thermococcales* alpha-amylase, is generally recognized as safe (GRAS), based on scientific procedures, when used as described below for the hydrolysis of edible starch in (1) the production of starch hydrolysis products such as high fructose corn syrup; and (2) the production of ethanol through starch fermentation for beverage use.

As such, this enzyme preparation is exempt from the premarket approval requirements of the Food, Drug, and Cosmetic Act.

(b)(6) 17 March 2003

David S. Hanselman, Ph.D.
Director of Regulatory Affairs, Environment, Health and Safety
Innovase LLC
5501 Oberlin Drive
San Diego, California 92121
858-352-4454
dhanselman@dow.com

2. Name and Address of the Notifier

Innovase LLC
5501 Oberlin Drive
San Diego, California 92121

3. Common or Usual Name of the Subject Substance

Alpha-amylase; α-amylase
(Also referred to as BD5088 α-amylase)

4. Conditions of Use

BD5088 α-amylase is intended for use as a processing aid to hydrolyze edible starch in the production of starch hydrolysis products such as high fructose corn syrup, other sweeteners, and ethanol. The enzyme preparation will be used at levels no higher than

necessary to achieve the intended effect, generally in the range of 500 grams of formulated enzyme preparation per metric ton of starch. This is equivalent to a concentration of 500 parts per million (0.5 kg ÷ 1000 kg x 10^6 ppm).

5. Basis for GRAS Determination

The GRAS determination for the subject enzyme preparation is based upon scientific procedures, as described in detail below.

6. Statement of Availability of Data and Information

The data and information that are the basis for Innovase's GRAS determination are available for review and copying by FDA at the offices of Keller and Heckman LLP, 1001 G Street, N.W., Washington, D.C. 20001. These documents will be sent to FDA upon request.

FDA is asked to address any interim communications and correspondence regarding this Notification to John B. Dubeck, attorney for the Notifier, at the following address / telephone number:

John B. Dubeck
Keller and Heckman LLP
1001 G Street, N.W.
Washington, D.C. 20001
202-434-4100
Dubeck@khlaw.com

We further ask that the Agency's final response letter to this Notification be addressed to Dr. Hanselman at the Innovase address provided above.

II) Notified Substance

II. Detailed Information about the Identity of the Notified Substance

1. Name: BD5088 Alpha-Amylase (α-Amylase)

2. Physical Description: amber liquid

3. Method of Manufacture

The BD5088 alpha-amylase preparation is made using generally known and accepted methods for the production of microbial enzymes (Aunstrup, 1979a; Aunstrup *et al.*, 1979b; Pariza and Foster, 1983). In addition, the BD5088 alpha-amylase preparation is manufactured in accordance with both Good Manufacturing Practices (GMPs) and the Organisation for Economic Co-operation and Development's criteria for Good Industrial Large Scale Practice (GILSP) (OECD, 1992).

A diagram of the manufacturing process is provided in Appendix 1.

3.a Production microorganism

Frozen glycerol stock cultures of the production microorganism, *P. fluorescens* strain DC88, are maintained as the source inoculum for enzyme production. The production microorganism is stable under these conditions. Each stock culture batch is examined for strain identity, purity, and enzyme-producing capability.

3.b Raw materials

Raw materials used in the fermentation and recovery process for BD5088 alpha-amylase are standard ingredients used in the enzyme industry (Aunstrup, 1979a, and Aunstrup *et al.* 1979b). The raw materials are of a purity and quality suitable for their intended use. No raw materials from animal sources are used. Each shipment of raw materials is evaluated by internal quality control standards to assure conformation with specifications.

The antibiotics tetracycline·HCl and kanamycin sulfate are added to the defined fermentation medium to maintain stability of the plasmids during enzyme production and to prevent contamination. No detectable antibiotic activity is found in the alpha-amylase product (see Composition and Specifications, Section II.4).

Isopropyl β-D-thiogalactopyranoside (IPTG) is added as an inducer for alpha-amylase production. It is present in the finished enzyme preparation at levels no higher than 100 ppm. Analytical testing has been conducted on starch-derived sweeteners produced with BD5088 α-amylase under typical processing and purification conditions to measure the amount of IPTG remaining in the finished sweetener. These analyses show at least a 57-fold reduction in the quantity of IPTG present in the finished sweetener attributable to

loss during processing and purification. Thus, assuming the maximum amount of IPTG is present in BD5088 α-amylase, 100 ppm, the corresponding maximum amount of IPTG in finished sweetener will be less than 1 ppb, as is shown below:

$$[\text{IPTG}]_{max} = 0.9\,\text{ppb IPTG} = \frac{500\,\mu\text{g BD5088}}{\text{g sweetener}} x \frac{100\,\mu\text{g IPTG}}{\text{g BD5088}} x \frac{1}{57} x \frac{1\,\text{g}}{1{,}000{,}000\,\mu\text{g}} x \frac{1000\,\text{ppb}}{\mu\text{g/g}}$$

A high purity enzyme (an endonuclease) and/or a food-grade mineral acid is optionally added as necessary to hydrolyze any residual antibiotic-resistance coding DNA that is not removed during the microfiltration step. The endonuclease is inactivated and is present at a concentration of less than 0.5 ppb in the enzyme preparation.

Suitable antifoaming agents used during manufacture and recovery of the alpha-amylase are of a suitable purity and appear on the Enzyme Technical Association's list of antifoaming agents (ETA, 1998).

3.c Fermentation process

The alpha-amylase production begins with a submerged fed-batch pure culture fermentation of the genetically modified *Pseudomonas fluorescens* strain DC88 (see Appendix 1). Process parameters that are routinely monitored during the fermentation include pH, aeration, enzyme activity, agitation, and temperature. During the fermentation, the broth is checked routinely for purity. Should evidence of a significant contamination exist, the broth is heat sterilized before being discarded.

After optimal alpha-amylase production is reached, the broth is pasteurized to ensure the production organisms are killed. The heat-treated broth is then transferred to the dissolution tank where the recovery process begins.

3.d Recovery process

In the dissolution tank, the pH of the broth is adjusted to the desired level with base to increase cell lysis and enhance enzyme extraction of the intracellular alpha-amylase, present as an inclusion body. Membrane microfiltration is used to separate cell debris from the solubilized enzyme fraction. Next, the enzyme is concentrated to the desired level of activity using ultrafiltration. The material is then transferred to a DNA treatment and formulation tank, where acid and/or endonuclease is optionally added as necessary to hydrolyze intact DNA. After DNA removal is complete, food-approved stabilizers, diluents, and preservatives are added to stabilize the product. The alpha-amylase enzyme preparation product is then stored in containers held in a temperature-controlled facility.

4. Composition and Specifications

Innovase's BD5088 alpha-amylase preparation complies with the General and Additional Requirements for Enzyme Preparations in the monograph on Enzyme Preparations in the fourth edition of the *Food Chemicals Codex* (National Academy of Sciences, 1996). In addition, the alpha-amylase preparation conforms to the General Specifications and Considerations for Enzyme Preparations Used in Food Processing outlined by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2001a).

The alpha-amylase enzyme preparation is formulated as a liquid with the following typical composition:

Component	Approximate Concentration
Total Organic Solids (TOS) [1]	3 ± 1 %
Glycerol (common name: glycerin)	20 to 30 %
Water	Balance

The enzyme preparation specifications are as follows. Additional information is available on the Product Specification Sheet provided in Appendix 2.

Specification	Permissible Level
Alpha-amylase enzyme activity, IAU/g: [2]	85% to 115% of declared units of activity
Total viable count/g:	Not more than 50,000 CFU
Total coliforms/g:	Not more than 30
Escherichia coli/25 g:	Absent
Salmonella spp./25 g:	Absent
Antibiotic activity:	Negative by test
Production organism:	Negative by test
Heavy metals (as Pb):	Not more than 30 mg/kg
Lead:	Not more than 5 mg/kg

The lead specification meets FCC and JECFA requirements. The heavy metals specification meets FCC requirements. The *E. coli* and antibiotic activity specifications meet JECFA requirements but are not included in FCC specifications. The total coliforms and *Salmonella* specifications both meet FCC and JECFA standards. The absence of the production microorganism in final product is an Innovase specification that is not included in FCC or JECFA specifications. Furthermore, the total viable count of the product is such that it will cause no increase in the total microbial count in the treated food over the level considered to be acceptable for the respective food, in accordance with FCC and JECFA general specifications.

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1) The TOS level in enzyme preparations is defined as 100% minus the sum of the moisture content, ash content, and any diluents or other substances added to the enzyme during formulation.

2) One Innovase Amylase Unit (IAU) is defined as the quantity of enzyme required to liberate one µmole of 4-nitrophenol from 5 mM 4-nitrophenyl-alpha-D-hexa-glucopyranoside over one minute at pH 7.0 and 75°C. The analytical method for enzyme activity can be found in Appendix 3.

III) GRAS Basis

III. Detailed Summary of the Basis for Notifier's GRAS Determination

1. Source Microorganism - Recipient strain

The microbial recipient strain used in construction of the alpha-amylase production strain is a natural bacterial isolate designated *Pseudomonas fluorescens* Biovar I strain MB101. Strain MB101, initially isolated in 1984 in Southern California from a lettuce leaf, grows in a temperature range of 25-33°C. This strain was morphologically, phenotypically and genotypically characterized.

The identity of strain MB101 was independently verified by Dr. I. J. Misaghi, Professor of Plant Pathology, University of Arizona, as *Pseudomonas fluorescens* Biotype A, which is equivalent under current taxonomic standards to *P. fluorescens* Biovar I. A report from Professor Misaghi with his conclusions is found in Appendix 4.

Phenotypic analyses of strain MB101 involved a variety of biochemical and microbiological characterizations, including API rapid NFT tests, tests for the production of levan, phenizine, and lecithinase, the ability to denitrify and liquefy gelatin, the utilization of trehalose and numerous other carbon sources, and fatty acid methyl ester cluster analysis.

Genotypic analyses were performed using the 16S rRNA genes of strains MB101 and other selected strains from the genus *Pseudomonas*. Genotypic analyses of the three hypervariable regions within the 16S rRNA genes corroborate the findings of the phenotypic analyses that identify strain MB101 as *P. fluorescens* Biovar I (Appendix 5).

The taxonomic designation of Strain MB101 is as follows:

Domain Bacteria
- Phylum BXII. *Proteobacteria*
  - Class III. *"Gammaproteobacteria"*
    - Order VIII. *Pseudomonadales*
      - Family I. *Pseudomonadaceae*
        - Genus I. *Pseudomonas*
          - Species *fluorescens*
            - Biovar I

2. Introduced DNA Sequences

High throughput screening of microbial DNA libraries was used to identify genes coding for alpha-amylases with desired pH optimum and temperature stability characteristics. Genes from three promising wild-type sources were recombined at non-conserved sites and the resulting alpha-amylases were characterized for desirable biochemical and productivity traits. One of these alpha-amylases, designated BD5088, was chosen for production optimization (Richardson *et al.,* 2002) (see Appendix 6).

Two plasmids were constructed and introduced into the recipient microorganism: plasmid pMYC5088, a 12.02 kb alpha-amylase expression plasmid; and plasmid pCN51lacI, a 7.06 kb auxiliary plasmid bearing the *lacI* repressor for tight regulation of the alpha-amylase gene. Plasmid maps are provided in Appendix 7.

Plasmid pMYC5088 is a self-replicating, non-conjugative, and non-mobilizable derivative of the well-known plasmid RSF1010 (Scholz *et al.*, 1989) that contains the following genetic material:

DNA Size	Source
6.32kb	from *Pseudomonas* cloning vector RSF1010 containing the replication regions
3.83kb	from plasmid RK2 containing the tetracycline resistance and regulatory genes (Pansegrau *et al.*, 1994)
0.28kb	promoter region that contains a DNA linker, *tac* promoter from pKK223-3 (Brosius and Doly, 1984), a synthetic enhancer, and a translation start sequence
1.34kb	BD5088 amylase gene
0.25kb	transcription terminator from *E. coli rrnBT1T2* (Brosius and Doly, 1984)

Plasmid pCN51lacI is also a self-replicating, non-conjugative, non-mobilizable plasmid that is a chimeric of the minimal self-replicating regions of the common pBR322 plasmid (Bolivar *et al.*, 1977) and the *Pseudomonas savastanoi* plasmid pPS10 (Nieto *et al.*, 1990).

It was found that a single copy of the *lacI* repressor gene on the *P. fluorescens* chromosome was not sufficient to ensure tight repression of the alpha-amylase gene, which caused poor growth and low enzyme yield. Introduction of plasmid pCN51lacI into the recipient strain resulted in optimal cell growth and enzyme production.

Plasmid pCN51lacI contains the following genetic material:

DNA Size	Source
1.26 kb	*E. coli lacI* gene fragment from pREP4 (Stuber *et al.*, 1990)
5.81 kb	pCN51 (Nieto *et al.*, 1990) that bears a kanamycin resistance gene

The DNA sequences of both plasmids are known. An exhaustive open reading frame analysis of both plasmids demonstrated that no dormant or silent genes were inadvertently introduced during plasmid construction.

3. Production strain construction

Plasmids pMYC5088 and pCN51*lacI* were electroporated into the recipient host *P. fluorescens* Biovar 1 strain MB101, resulting in the production microorganism *P. fluorescens* Biovar 1 strain DC88.

4. Stability of the introduced DNA sequences

The plasmids introduced into the recipient microorganism and contained in the production microorganism remain as extrachromosomal DNA, *i.e.*, the plasmids are not integrated into the chromosome. The plasmids remain stable in the production microorganism during alpha-amylase manufacture as shown by two methods: plating and counting of colonies on selective and non-selective media; and by restriction digests of plasmid DNA isolated from shakeflask cultures which had been sub-cultured in non-selective medium for up to 45 generations.

A suggested criterion of the Organization of Economic Cooperation and Development (OECD) recombinant DNA Good Industrial Large-Scale Practice (GILSP) is that only poorly mobilizable plasmid vectors are used for large-scale production of recombinant products to enhance biological containment (OECD 1986, 1992). The mobilization potential of plasmids pMYC5088 and pCN51lacI was examined by both DNA sequence analysis and experimentation. It was found that the plasmids in the alpha-amylase production strain are not self-transmissible and are poorly mobilizable. Without the presence of a helper plasmid, the transmission of both plasmids was significantly lower than the 10^{-8} recommended by NIH guidelines (NIH, 2001). With a helper plasmid, the experimental mobilization frequencies of pMYC5088 and pCN51lacI were on the same order, 10^{-6}-10^{-8} per recipient, as the NIH certified *Pseudomonas* plasmids pKT262, pKT263 and pKT264 (Bagdasarian *et al.*, 1981).

5. Enzyme Identity

5.a Identity

The subject of this GRAS Notification is an alpha-amylase as defined by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB, 1992). The enzyme is responsible for the endohydrolysis of 1,4-α-D-glucosidic linkages in polysaccharides containing three or more 1,4-α-linked D-glucose units.

Classification:	α-amylase
Systematic name:	1,4–α-D-glucan glucanohydrolase
Alternative Names:	alpha-amylase; glycogenase; endoamylase
IUB No.:	3.2.1.1
CAS No.:	9000-90-2
EINECS No.:	232-565-6
Specificity:	1,4-α-glucosidic linkages in amylose and amylopectin
Molecular Weight:	49,770 kDa

5.b Amino acid sequence

The alpha-amylase designated BD5088 is a hybrid enzyme derived from three wild-type archaeal alpha-amylases. The hybridized enzyme was selected for commercialization due to its stability and activity at low pH, high temperature, and low calcium concentration. The complete amino acid sequence for BD5088 alpha-amylase is presented in Appendix 10, Table 1.

5.c Sequence comparison to other alpha-amylases

The amino acid sequences for numerous alpha-amylases from various sources have been published (Nakajima, R *et al.*, 1986; Henrisaatt, 1997). Data show that every alpha-amylase, even from very different phylogenetic origins, has four conserved homologous regions that correspond to the active and substrate-binding sites of the enzyme. Appendix 8a compares the amino acid sequence of the BD5088 alpha-amylase to six other alpha-amylases from various sources. BD5088 alpha-amylase contains every highly-conserved region common to all alpha-amylases, and is similar at the amino-acid level to each of these known amylases.

The amino acid sequence of the BD5088 alpha-amylase was also compared to those from several naturally-occurring archaeal alpha amylases whose sequences have been published: *Pyrococcus woesi* (Koch *et al.*, 1991), *Pyrococcus* sp. KOD1 (Tachibana *et al.*, 1996), *Pyrococcus furiosus* (Dong, *et al.*, 1997), *Thermococcus* strain Rt3 (Jones *et al.*, 1999), *Thermococcus hydrothermalis* (Leveque *et al.*, 2000a), and *Thermococcus profundus* (Chung *et al.*, 1995; Lee *et al.*, 1996). This information is presented in Appendix 8b. Alignments of the thermophilic archaeal alpha-amylase amino acid sequences demonstrate at least 85% homology to BD5088 alpha amylase. The characteristics of the thermophilic archaeal alpha-amylases have been summarized in a recent review by Lévêque *et al.* (2000b). In brief, these enzymes fall into one family, designated family 13, which for the most part do not differ from their mesophilic counterparts in molecular weight or amino acid composition.

From the information provided above, it is apparent that the BD5088 is highly related to the naturally-occurring thermophilic archaeal alpha-amylases. Further, BD5088 contains all highly conserved regions common to other sequenced alpha-amylases from archaeal, bacterial, fungal, plant and mammalian sources.

5.d Functional Properties

Several experiments were performed to show the technical effects of BD5088 alpha-amylase on starch and to compare its use to other commercially available alpha-amylases. Starch hydrolysates treated with alpha-amylase were examined for resulting carbohydrate spectra. Also, glucose syrups were produced and the yield of glucose and other oligosaccharides was examined (see Appendix 9). The results confirm that BD5088 has

catalytic and functional properties similar to other commercial alpha-amylases with respect to starch hydrolysis.

6. Absence of the production organism in the enzyme preparation

The absence of the production organism in the commercial product is an established specification (see Section II.4 above). Because the production organism is not present in commercial product, it does not end up in food.

7. Absence of transformable DNA in the enzyme preparation

An examination for the presence of transformable DNA in finished enzyme preparation was made. No plasmid DNA capable of transformation was detected.

8. Antibiotic resistance genes

As discussed in Section 2 above, the production microorganism used for the manufacture of the alpha-amylase enzyme preparation carries two antibiotic resistance markers, one for kanamycin and the other for tetracycline. The purification process ensures that the food-grade alpha-amylase enzyme preparation does not contain functional copies of the antibiotic resistance coding genes. Microfiltration through a membrane results in the rejection of significant amounts of DNA from the soluble enzyme fraction. Acid and/or enzymatic (endonuclease) hydrolysis is used if needed to ensure removal of intact full-length antibiotic resistance DNA. This is confirmed via a Southern hybridization procedure that has a detection limit of approximately 950 parts per trillion (ppt or pg/g).

9. Applications and Intake Estimate

9.a Foods in which used and purpose of use

The enzyme of this notification is used for the hydrolysis of edible starch in the production of starch hydrolysis products and alcoholic beverages.

In the starch industry, thermostable bacterial alpha-amylases similar to the enzyme of this notification are used to thin gelatinized starch, which may then be subjected to further processing with other suitable enzymes. The source of starch may be any of several grains or root-based crops. In practice, starch-derived sweeteners produced and/or marketed in the United States are almost all derived from corn. Additional enzyme treatment will consist of hydrolysis with glucoamylase or other debranching and/or saccharifying enzymes to produce starch hydrolysates of desired compositions. Typical products include, but are not limited to: corn syrup, dextrose (the industry name for products composed of essentially D-glucose), high fructose corn syrup, LB 9 (a low fructose syrup), maltose syrup (which includes brewer's syrup) and maltodextrin.

BD5088 alpha-amylase will also be used in the production of ethanol for alcoholic beverages. Hydrolysis of starch provides an increased level of fermentable sugars which results in increased ethanol yields and reduced residual carbohydrate levels.

9.b Mode of action

Alpha-amylases are responsible for the endohydrolysis of 1,4-α-D-glucosidic linkages in polysaccharides containing three or more 1,4-α-linked D-glucose units. Innovase's alpha-amylase offers advantages over conventional heat-stable enzymes, including its tolerance to low pH and low calcium ion concentration, and high-temperature stability.

9.c Level of use

The recommended dosage of Innovase's alpha-amylase is 500 grams of formulated enzyme preparation per metric ton of starch (500 ppm). In other words, 0.5 kg enzyme preparation, which contains roughly 3% total organic solids, is added to 1000 kg starch.

9.d Enzyme residues in food ingredients

The enzyme is expected to be present, if at all, in the finished HFCS, glucose, dextrose, or other starch-derived sweetenersat levels below the limit of sensitivity of currently-available analytical methodology. This is because of the repeated purification steps to which the sweeteners are subjected. The processing of corn starch, for example, to produce ingredients such as HFCS, glucose syrup, and crystalline glucose, includes a series of purification steps intended to remove unwanted organic components. These steps include multiple cation- and anion-exchange cycles, as well as activated-carbon treatment.

The *per capita* intake of all corn sweeteners in the United States is 104 g/person/day according to statistics made available by the Corn Refiners Association.[3] More specifically, total corn sweeteners (including HFCS, glucose, and dextrose) were consumed at a rate of 84 pounds per person annually in 2001. This corresponds to 104 grams/person/day.

Analytical testing has shown that the enzyme cannot be detected, at a limit of detection of 2.4 ppb (ng/g), as measured by sandwich ELISA (enzyme-linked immunosorbent assay), in glucose syrup processed using BD5088 α-amylase. A rough, *per capita* intake estimate may be calculated using the following additional assumptions and information: (1) residues of the inactivated enzyme may be present in starch-derived sweeteners at one-half the limit of detection (LOD), 1.2 ppb; (2) other components of the TOS are removed during purification at the same rate at which the enzyme is removed; and (3) the enzyme itself comprises at least 10% of the TOS.

3) www.corn.org/web/percapsw.htm, citing as its source the U.S. Department of Agriculture's Economic Research Service.

Thus, when these conservative assumptions are taken into account, the *per capita* intake of BD5088 TOS associated with starch-derived sweeteners is approximately **1.2 µg/person/day, or 0.4 ppb in the diet**, assuming 3000 grams of liquid and solid food per day. These derivations are as follows:

$$\textit{per capita}\ \text{intake} = \frac{1.2\ \mu\text{g TOS}}{\text{person}\cdot\text{day}} = \frac{104\ \text{g sweetener}}{\text{person}\cdot\text{day}}\ x\ \frac{1.2\ \text{ng BD5088}}{\text{g sweetener}}\ x\ \frac{100\ \text{parts TOS}}{10\ \text{parts BD5088}}\ x\ \frac{\mu\text{g}}{1000\ \text{ng}}\ ,$$

$$\textit{per capita}\ \text{dietary concentation} = 0.4\ \text{ppb TOS} = \frac{1.2\ \mu\text{g TOS}}{\text{person}\cdot\text{day}}\ x\ \frac{1000\ \text{ppb}}{\mu\text{g/g}}\ x\ \frac{\text{person}\cdot\text{day}}{3000\ \text{g}}\ .$$

A heavy consumer of starch-derived sweeteners can be expected to consume some amount more than the *per capita* intake, 104 g/person/day. A factor of 10 is sometimes applied to the *per capita* intake of a substance to approximate consumption among heavy consumers. In the case of starch-derived sweeteners, which are distributed very broadly throughout the food supply, a 10-fold increase in *per capita* consumption appears highly exaggerative of heavy consumption, at more than 1000 g/person/day. We propose that a factor of three times the *per capita* intake is more appropriate for approximating heavy consumption of starch-derived sweeteners, at 312 g/person/day.

The additional 3x *per capita* safety factor yields an estimated intake of BD5088 TOS of 3.6 µg/person/day, or 1.2 ppb in the diet. In either case, it is clear that an abundant safety factor exists between the estimated daily intake and the NOAEL observed in the 90-day rat feeding study. The NOAEL, 890 mg/kg b.w., is **44,500,000 times greater** than the *per capita* estimated intake, 1.2 µg/person/day. This can be expressed as 0.00002 mg/kg b.w. for a 60-kg adult. For heavy consumers, the safety factor is divided by 3, and yet it is still more than 14.8 million times the calculated intake:

$$\text{Safety Factor}_{\text{Heavy}} = 14{,}800{,}000 = \frac{60\ \text{kg}\cdot 890\ \text{mg TOS}}{\text{person}\cdot\text{day}\cdot\text{kg}}\ x\ \frac{\text{person}\cdot\text{day}}{3\cdot 1.2\ \mu\text{g TOS}}\ x\ \frac{1000\ \mu\text{g}}{\text{mg}}$$

It should be noted that even if the removal of the BD5088 α-amylase during purification of the sweeteners were discounted, the amount of enzyme remaining in the finished sweetener could not exceed the amount added, 15 ppm (calculated as TOS), which would correspond to approximately 5 mg/person/day for heavy consumers. This level of exposure represents less than one ten-thousandth of the NOAEL and still yields a large safety factor. The derivation of these estimates are as follows:

$$\textit{per capita}\ \text{intake}_{\text{Max}} = \frac{4.7\ \text{mg TOS}}{\text{person}\cdot\text{day}} = \frac{3\cdot 104\ \text{g sweetener}}{\text{person}\cdot\text{day}}\ x\ \frac{500\ \mu\text{g formulation}}{\text{g sweetener}}\ x\ \frac{3\ \text{parts TOS}}{100\ \text{parts formulation}}\ x\ \frac{\text{mg}}{1000\ \mu\text{g}}$$

$$\text{Safety Factor}_{\text{Min}} = 11{,}400 = \frac{60\ \text{kg}\cdot 890\ \text{mg TOS}}{\text{person}\cdot\text{day}\cdot\text{kg}}\ x\ \frac{\text{person}\cdot\text{day}}{4.7\ \text{mg TOS}}\ .$$

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In addition to its use in the production of starch-derived sweeteners, BD5088 α-amylase will be used in early stages of the production of ethanol from corn or grain for use in alcoholic beverages. Like other α-amylase enzymes, BD5088 will be used to hydrolyze starch to provide an increased level of fermentable sugars, which results in increased ethanol yields. When ethanol is produced by fermentation in this manner, it is purified through distillation. Because ethanol is relatively low boiling (79°C), no measurable amount of BD5088 α-amylase will be present in the purified ethanol distillate.

10. Safety Testing

Safety studies performed on recipient strain *P. fluorescens* MB101 and the BD5088 alpha-amylase enzyme preparation are the subject of a peer-reviewed scientific publication by Landry *et al.* (2003). This paper is found in Appendix 10. These and other relevant safety studies are briefly summarized below.

10.a Microorganism

10.a.i Published Pathogenicity Studies

The U. S. Environmental Protection Agency (EPA) conducted two *in vivo* studies to evaluate the possible health concerns associated with the use of *P. fluorescens* as a microbial pest control agent (George *et al.*, 1999; George *et al.*, 2000). The results of the study by George and coworkers (1999) demonstrated that *P. fluorescens* was eliminated from the lungs, cecum, small and large intestine by 2 days post-treatment. *P. fluorescens* was detected in the liver and mesenteric lymph node 3 three hours after treatment but had disappeared completely from these tissues within 2 days of treatment. No mortality in the mice was noted at bacterial concentrations as high as 5.0×10^8 CFU/mouse although some mortality was observed at excessively high ($\sim 10^9$/mouse) bacterial concentrations.

In the second study, male CD-1 mice were treated perorally with an average dose of 1.78×10^8 CFU of *P. fluorescens* per mouse (George *et al.*, 2000). *P. fluorescens* was recovered in the intestinal tract after 3 hours but was completely cleared after the first day. *P. fluorescens* was detected in lungs, intestinal tract (small, large, cecum), mesenteric lymph node, spleen, and liver three hours after treatment but had completely cleared from all organs and tissues 2 days after treatment. At the completion of the study all mice treated with *P. fluorescens* appeared healthy and conventional indicators of morbidity, such as ruffled fur, lethargy, weight loss, conjunctivitis, were not present.

10.a.ii Pathogenicity and Toxigenicity Testing of *P. fluorescens* Biovar 1 Strain MB101

The pathogenic and toxigenic potential of orally administered *Pseudomonas fluorescens,* Biovar I, strain MB101 was evaluated in Balb/c mice. Strain MB101

is the parental strain of the *P. fluorescens* that is used to produce BD5088. Test material was administered by oral gavage in a suspension of bacteria formulated to contain 6 x 10^8 or 1x 10^8 CFU per mouse. Suitable control groups were included for comparison. Mice were held for up to 21 days, with daily general observations of health. Subgroups of six bacteria-treated mice underwent necropsy on days 2, 4, and 7; and liver, spleen, mesenteric lymph node (MLN), large bowel, small bowel and cecum were sampled for measuring bacteria. A subgroup of control mice underwent necropsy on day 1.

The ability of the test strain to infect mice was measured by the recovery of the dosed strain from selected organs and tissues. Oral exposure of *P. fluorescens* resulted in detectable levels of pseudomonads in all mice examined, although significant heterogeneity was noted on day two in the number of CFU recovered on the selective medium within each subgroup of mice. No mortality was observed over a 21-day period following oral administration. Infection with *P. fluorescens* did not result in any clinical signs of morbidity such as ruffled fur or lethargy during the 21-day period. The animals appeared healthy and did not exhibit any weight loss, as the body weights of the infected animals were not significantly different from the uninoculated controls. Oral administrations of high doses of *P. fluorescens*, Biovar I, strain MB101, resulted in the translocation of the test strain to the MLN, spleen and liver of adult male Balb/c mice. The test strain did not appear to be infectious and the organisms were eliminated from these tissues within four days of exposure. Microorganisms capable of growth on Pseudomonas Isolation Agar (PIA) plates were also detected in the bowels and ceca. Elimination of the test strain from the bowels and cecum was difficult to discern since the normal microbial flora of the uninoculated control mice produced a high level of background CFU on PIA plates.

Bacterial clearance requires an intact and functional immune system that incorporates a cascade of immune responses. In this animal model, bacterial clearance provided an indication of the interaction between the potential pathogenicity of the invading microorganism and the total host immune capability. Similar results were reported by George *et al.* (2000): there was rapid clearance of *P. fluorescens* from the MLN, spleen and liver in male CD-1 mice treated orally with high levels (~10^8 CFU/mouse) of this microbial agent. George *et al.* noted some mortality at extremely high levels (approximately 10^9 bacteria/mouse) following *intra nasal* administration.

The full study report describing the MB101 recipient strain pathogenicity study was provided to Dr. Stanley Falkow, Professor of Microbiology and Immunology at the Stanford University School of Medicine, who is among the world's leading experts in bacterial pathogenesis. At the completion of his review, Professor Falkow provided a letter, included as Appendix 11, in which he states:

"... it is my professional judgement the strain of *P. fluorescens* you propose to utilize is non-pathogenic and does not represent an infectious threat of any kind to humans who might come into contact with these bacteria. ... this microbe you plan to use represents no health risk to your workers or to those who may use it or products derived from it in the future".

10.b Enzyme Product

10.b.i 5-Day Acute Oral Gavage in Rats

Methods: BD5088 α–amylase preparation (0 and 790 mg TOS/kg/day) was administered by oral gavage (10 ml/kg) to 5 female Fischer rats for 5 consecutive days. Parameters evaluated included daily cage-side observations, detailed clinical observation, urinalysis, body weights, feed consumption, hematology, clinical chemistry, organ weights, and gross and histopathologic observations. Urine was collected prior to necropsy and was evaluated for volume, color, appearance, specific gravity and pH, bilirubin, glucose, proteins, ketones, blood and urobilinogen.

Results: Rats given the test material at a dose of 790 mg TOS/kg/day had a microscopic alteration in the intestinal tract, characterized by a loss of heterochromatin from epithelial nuclei, giving the cells a vesicular appearance. This occurred in most of the treated rats and most prominently in the rectum. A slight inflammatory reaction in the nasal tissues / turbinates was considered to be a result of the inadvertent reflux of test material into the nasal cavity during the gavage procedures. There were no other treatment-related changes based on observations, body weights, feed consumption or clinical and anatomic pathology.

10.b.ii 14-Day Range Finding Gavage in Rats

Methods: Groups of 10 rats per sex were given the test material at doses of 0, 71, 220, 710 or 2,200 mg TOS/kg/day by gavage for 14 consecutive days. Parameters were evaluated as described above (5-day oral gavage study) but with weekly clinical observations and additional ophthalmological observations. The eyes of all animals were examined by a veterinarian pre-exposure and prior to the scheduled necropsy using indirect ophthalmoscopy. Complete histopathological evaluations were carried out on all high-dose and control animals; based on results observed in these animals, liver and nasal tissues from animals dosed at the intermediate levels were also evaluated.

Results: Feed consumption was slightly decreased among male rats given the test material at doses of 710 and 2200 mg TOS/kg/day; males in the higher dose group, 2200 mg/kg/day, had statistically identified decreases in mean body weights, with a mean value 8.2% less than control at termination. Statistical

increases in several relative organ weights (2200 mg/kg groups) were considered secondary to the decrease in body weight. There were several isolated observations of noisy respiration in rats receiving the test material at doses of 710 and 2200 mg/kg/day: this was considered to be a secondary effect of probable regurgitation. At the top dose of 2200 mg/kg/day, terminal serum cholesterol was increased and a very slight lipid vacuolation primarily of periportal hepatocytes was observed. The degree of vacuolation was not considered to be adverse.

Local suppurative inflammation of the nasal mucosa was seen in the majority of males given 220 mg/kg and greater and of females given 71 mg/kg and greater. In rats given the top dose, nasal tissues of 3 males and 4 females had normal appearance. This focal response and lack of effect in some top dose rats was consistent with local irritation due to the gavage procedure, whereby small quantities of test material are likely to reflux into the nasal tract when the intubation needle is withdrawn. A small increase in mean spleen weight of female rats given 71, 710 and 2200 mg/kg/day was not considered to be treatment related due to the small magnitude (relative increase of 11% in top dose group), the range in comparison with historical values, and the lack of corresponding histopathology. There were no other treatment-related changes based on observations, organ weights or pathology. Based on the decreased body weight in males given the test material at 2200 mg/kg/day, the NOAELs were 710 mg/kg/day in males and 2200 mg/kg/day in females.

10.b.iii 90-Day Subchronic Gavage in Rats

Methods: Groups of 20 male and 20 female Fischer 344 rats were administered 0, 89, 270 or 890 mg BD5088 α–amylase/kg/day (as TOS) via oral gavage for 13 weeks to evaluate the potential for systemic toxicity. Parameters examined were as described above (5-day oral gavage study). In addition, cage-side clinical observations, weekly detailed clinical observations, ophthalmological exams, weekly body weights, weekly feed consumption, week 12 urinalysis, hematology, clinical chemistry and organ weights were recorded.

Results: Three rats died during the study (two males in the 89 mg/kg/day dose group and one female in the 890 mg/kg/day dose group), with lung inflammation and edema noted in the pathology examination. Based on these findings, the deaths were attributed to aspiration of the test material due to gavage error. The only treatment-related clinical observations were occasional noisy respiration, also considered to be gavage related.

Some rats from both sexes and all treatment groups had very slight to slight chronic active inflammation of the nasal mucosa (respiratory and/or olfactory). Some of the male rats given 270 or 890 mg/kg/day and one female given 270 mg/kg/day had mucopurulent exudates in the nasal cavities. Additionally, some of the rats of both sexes and from all treatment groups had very slight to slight, focal to multifocal, subacute to chronic inflammation of the lung. These nasal

and lung effects were interpreted to be related to reflux of the test material into the nasal cavity and/or aspiration into the lung. Because the enzyme preparation was made with a Gram negative production organism, it contained lipopolysaccharide (endotoxin). Inhalation or instillation of endotoxins can cause inflammation of the respiratory tract. The presence of endotoxins in the final formulation is not expected to significantly increase the levels of endotoxins in the final food products above background levels. Moreover, endotoxins do not demonstrate toxicity when administered via the oral route and would not be considered a safety issue for food processing enzymes. A brief background discussion on endotoxins is provided in Appendix 13.

There were three measurements of weekly feed consumption in 890 mg/kg/day treated male rats that were statistically decreased; these were considered to be related to the nasal inflammation. Exudative nasal inflammation was associated with decreased feed consumption and increased circulating neutrophils in individual males given 890 mg/kg/day. Feed consumption was not consistently affected in the treatment groups.

Myeloid cell hyperplasia was observed in three males (890 mg/kg/day); this was considered secondary to the respiratory inflammation. Increased observations of white blood cells in urine of males given 270 or 890 mg/kg/day were noted (not a statistical comparison). There was no corresponding urinary or renal histopathology. Treatment-related pathologic effects were limited to the respiratory tract. Body weights were not affected to a degree that was statistically identifiable, although feed consumption was affected at times in individual rats. Although some rats from all treatment groups had a degree of local respiratory tract inflammation, there were no adverse direct systemic effects considered to be related to the oral route of administration.

Based on the results of this study, the NOAEL for BD5088 α-amylase enzyme preparation (TOS basis) in Fischer 344 rats of either sex was considered to be 890 mg/kg/day, the top dose administered.

10.b.iv Follow-Up 14-Day Dietary Feeding Study in Rats

To investigate the significance of effects noted in the respiratory tracts of animals in the course of gavage studies, groups of 20 male and 20 female Fischer 344 rats were administered 0 or 360 mg (males) or 310 mg (females) BD5088 α-amylase/ kg/day (as TOS) in the diet for 14 days.

Methods -- The test material was pre-mixed in a corn oil slurry, then mixed in the diet so that the final corn oil concentration was 1 to 2 %. This procedure was used to minimize potential dusting that could occur when the lyophilized BD5088 preparation was mixed in the diet.

Cage-side clinical observations, weekly body weights and weekly feed consumption were evaluated. Blood samples for hematology were collected prior to necropsy. Gross necropsies and histopathology of the entire respiratory tract (nasal tissues to lung) were conducted on all rats. Liver samples were also examined histologically as some effects had been observed in the 5-day gavage study.

Results -- Administration of BD5088 α–amylase enzyme preparation in the diet at a level of 360 (males) or 310 (females) mg/kg/day (TOS basis) did not result in any treatment-related effects on body weights, food consumption, or cage-side clinical observation. No treatment-related effects on hematology, including neutrophils, were observed. Further, gross and histopathologic examination of the respiratory tract and liver did not reveal any macroscopic or microscopic treatment-related changes in these tissues.

In the 14-day and 90-day gavage studies discussed above, localized inflammatory lesions were observed in the nasal mucosa and/or lungs of treated animals. These lesions were interpreted to be due to reflux and/or aspiration of the test material into the nasal cavity or lungs during the course of repeated oral gavage dosing. Hence, these results were interpreted to be not relevant to identifying the effects of BD5088 α-amylase when it is ingested as a component of the diet. In this feeding study, such inflammatory changes of the lungs and nasal cavity were not observed, thus confirming that the inflammatory effects observed in the previous studies were due to the gavage route of administration and are not relevant to ingestion of BD5088 α–amylase enzyme preparation residues in food.
Dr. Jack R. Harkema, an expert pathologist and Professor of Comparative Pathology at Michigan State University, examined respiratory-tract slides from the 14-day dietary and 13-week gavage studies. Professor Harkema, who is internationally recognized for his research on the effects of endotoxins in mammals, confirmed that the observed respiratory-tract inflammation was similar to those found when endotoxins were administered to rodents. His histopathology review can be found in Appendix 12.

10.b.v – Studies on Genotoxicity

BD5088 α-amylase has been tested to investigate its genotoxic potential in a range of *in vitro* and *in vitro* short-term genotoxicity screening assays. These include a mouse lymphoma forward mutation assay, a mouse bone marrow micronucleus test, a chromosomal aberration assay using rat lymphocytes, and bacterial mutagenicity assays in *Salmonella* (*i.e.*, Ames test). The results of these studies are summarized in the following table. A more complete description of these studies is provided in Appendix 10.

Test System	Test Object	Concentration of BD5088	Results
Mouse Lymphoma Forward Mutation Assay [a]	L5178Y $TK^{+/-}$ cells	125-5000 µg/ml	Negative
Mouse Bone Marrow Micronucleus Test	male CD-1 mice	0 - 2000 mg/kg b.w.	Negative
Chromosomal Aberration Assay Using Rat Lymphocytes	Lymphocytes from Sprague-Dawley rats	0 - 5000 µg/ml	Negative
Ames Test [a]	*S. typhimurium* TA98, TA100, TA1535, TA1537, *E. coli* WP2uvrA	up to 5000 µg/ml	Negative

[a] With and without S-9 metabolic activation.

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IV) Safety Evaluation

IV. Safety Evaluation Discussion

1. Alpha-Amylase

1.a Safety of food enzymes as processing aids

Pariza and Foster (1983) and Pariza and Johnson (2001) addressed the issue of food processing enzyme safety and concluded that enzymes, *per se*, that are used or likely to be used in food processing are inherently nontoxic, thus the safety evaluation should focus on possible contaminants which could be present in the final enzyme preparation. These authors suggest that the safety of the production strain should be the primary consideration when evaluating enzyme safety.

The fact that enzymes are a biological necessity for all living organisms offers a high degree of safety assurance for these proteins. Enzymes have always been present as natural components of food, thus the history of human exposure to enzymes is as old as human life itself. The application of enzymes to food processing has developed from traditional recipes such as those for fermented foods whose preparation is older than recorded history.

1.b History of use of alpha-amylase in foods

Today, enzymes are used in food to accomplish a variety of purposes. A major use has been and still is, for the hydrolysis of starch. Beckhorn *et al.* (1965) reported that bacterial alpha-amylase has been in common use since 1929 to control the viscosity of chocolate syrup. (While the stated use for this example is viscosity control, the functional use is for starch hydrolysis.) Furthermore, these authors report that alpha-amylase has been used for the liquefaction of cooker mash in brewing and distilling since 1936, and for the modification of cereal starches to improve characteristics of precooked cereals since 1952. These examples illustrate the extensive history of use of alpha-amylases in a variety of foods.

During this period when bacterial alpha-amylases came into common use, the source organism was *Bacillus subtilis* (Aunstrup, 1979; Peltier and Beckord, 1945). Later, thermostable alpha-amylases from *Bacillus licheniformis* and *Bacillus stearothermophilus* were developed. In 1973, a GRAS petition which proposed the affirmation of the GRAS status of mixed carbohydrase and protease preparations from *B. licheniformis* was accepted for filing by the FDA (FDA, 1973). In 1983, the FDA affirmed this enzyme preparation as GRAS (FDA, 1983b). The affirmation for GRAS status of *B. stearothermophilus* alpha-amylase preparations was filed with the FDA in 1983 (FDA, 1983a) and the FDA affirmed the GRAS status of this enzyme preparation in 1995 (FDA, 1995a).

More recently, three modified thermostable alpha-amylases have been determined to be GRAS and commercialized for use in the starch and alcoholic beverage industries (Novo Nordisk, 1999a, 1999b; Novozymes, 2001). FDA issued Agency Response Letters to the

GRAS Notifications for these three enzyme preparations and indicated that it had no questions at the time regarding the GRAS determinations.

From the information provided above, it is apparent that alpha-amylases have a long history of safe use in food processing.

1.c Substantial equivalence

The concept of substantial equivalence as it applies to food safety assessments has been the topic of much recent discussion (Berkowitz and Maryanski, 1989; OECD, 1993; JECFA, 1996; Jonas *et al.*, 1996; Kessler *et al.*, 1992; FDA, 1995b; FDA, 1997b; Maryanski, 1995). Among these experts, there is general consensus that, if a food ingredient is substantially similar to an existing food ingredient known to be safe, then generally no further safety considerations other than those for the existing ingredient are necessary. In several instances, the FDA has stated that enzyme proteins shown to be substantially equivalent to enzymes known to be safely consumed but having differences in specific properties due to change in the enzyme amino acid sequence by natural selection, chemical modification, or site-directed mutagenesis would not raise safety concerns (Kessler *et al.,* 1992; FDA, 1997b). This concept has been directly applied to food processing enzyme preparations (FDA, 1995b; FDA, 1993; FDA, 1997a; Novo Nordisk, 1999a and b; Novozymes, 2001).

Recently, Novozymes North America, Inc. determined the lipase enzyme preparation from *Aspergillus oryzae* carrying a gene constructed from a modified *Thermomyces lanuginosus* lipase gene and a portion of the *Fusarium oxysporum* lipase gene, to be GRAS (Novozymes, 2002). In reply to the GRAS Notice, the FDA has issued an Agency Response Letter in which it is stated that the agency has no questions regarding the conclusion that the enzyme preparation is GRAS under the intended conditions of use. Like the BD5088 alpha-amylase, the lipase of the enzyme preparation is a hybrid enzyme.

The information presented in Section III.5 demonstrates the substantial equivalence of the BD5088 alpha-amylase to other alpha-amylases. Specifically, the BD5088 alpha-amylase contains the four highly conserved regions that are common to all alpha-amylases and is similar at the amino acid level to these known amylases.

Further, the BD5088 enzyme was compared to two commercially available alpha-amylase enzyme preparations (Termamyl® LC and Termamyl SC) in the production of dextrose syrup. The alpha-amylases were used to liquefy starch under typical conditions (35% d.s., 105-106°C, 5-6 minutes for primary liquefaction and 95-96°C, 2 hours for secondary liquefaction) with two exceptions: the Termamyl preparations were dosed at pH 5.8 with 20 ppm added calcium, while the BD5088 preparation was tested at pH 4.8 and 10 ppm added calcium.

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The results in Appendix 9 show that 1) the reducing sugar formation followed a similar course for all three enzymes during liquefaction; 2) all three enzymes produced the same oligosaccharides during liquefaction; and 3) the glucose yields and carbohydrate profiles were equivalent after saccharification using the liquefaction hydrolysate made from each of the three enzymes. Thus, the action of the BD5088 alpha-amylase on starch is equivalent to that of the two commercially available alpha-amylases, but offers the advantage of less base and calcium addition.

Pariza and Johnson (2001) state that it is unlikely that changes to an enzyme which improve its desired function (ex. changing pH optimum, increasing thermal stability, reducing the requirement for cofactors such as metal ions, and stabilizing the enzyme against oxidation) will result in creation of a toxic protein. Nonetheless, they suggest that it is prudent to assess this very small theoretical risk by conducting limited toxicological tests on the engineered enzyme. Toxicological testing with the alpha-amylase preparation verifies the safety of the product (see Section III.10.b). Thus, one can conclude that the BD5088 alpha-amylase is substantially equivalent to other alpha-amylases and is safe for use in food.

1.d Allergenic Potential Assessment

In assessing the safety to human health of genetically modified proteins used in food processing, the allergenic potential of the protein should be assessed (JECFA, 2001a). A comparison of the amino acid sequence of the modified protein to known protein allergens is one step in a multilevel decision tree to assess allergenic potential (Metcalfe *et al.*, 1996).

A sequence evaluation scheme based on those formulated by Gendel (1998) and by the Joint FAO/WHO Expert Consultation (2001b) was used to assess the similarity of the BD5088 alpha-amylase to known protein allergens. It has been suggested that an immunologically significant sequence identity requires a match of at least eight contiguous identical amino acids or 35% identity over eighty amino acid residues. In this study, no such sequence identity was detected for BD5088 alpha-amylase; therefore, based on amino acid sequence data, BD5088 alpha-amylase would not be projected to have allergic potential.

The Association of Manufacturers and Formulators of Enzyme Products (Amfep) Working Group on Consumer Allergy Risk from Enzyme Residues in Food published a thorough review of the published literature on the possible role of enzymes in food allergies (Amfep, 1998). The Group concluded that there were no reports documenting that consumption of food with residual enzymes has caused sensitization or induction of allergic reactions in consumers. Although cases have been reported in which consumers developed symptoms after eating bread made with α-amylase, these consumers first had occupational (baker's) allergy to the enzyme, which apparently caused the sensitization. The possibility that cross-reactions might occur was tested in a double blind placebo controlled food challenge study on 17 people allergic to *Aspergillus* molds (Cullinan *et al.*, 1997). All subjects had antibodies to *Aspergillus fumigatus,* and 6 reacted to skin

prick tests with enzymes produced from *A oryzae* or *A. niger*. However, when challenged with bread containing these two enzyme preparations, no reactions were observed during a 24 hour monitoring period as compared to the placebo.

2. Production microorganism

The safety of the production organism is the prime consideration when assessing the probable degree of safety of an enzyme preparation intended for use in food (Pariza and Foster, 1983; Pariza and Johnson, 2001). If the enzyme production organism is nonpathogenic and nontoxigenic, and the enzyme is made according to current good manufacturing practice (cGMP), then one can assume the food ingredient produced from the production microorganism is safe to consume (IFBC, 1990).

Pseudomonas fluorescens is a common saprophyte that inhabits plant rhizosphere and phyllosphere environments (OECD, 1997). The microorganism has been used in recent years in a variety of industrial applications, including for ice nucleation (for snowmaking and minimization of frost damage to plants) (Warren, 1987; Lindlow and Panopoulos, 1988; Wilson and Lindlow, 1993), to produce biological pesticides (Herrera *et al.*, 1994; Mycogen, 1991), and in the control of diseases in the phyllosphere of plants (Wilson and Lindlow, 1993).

Strains of *P. fluorescens* are commonly found on plant surfaces, as well as decaying vegetation, soil, and water (Bradbury, 1986). The ubiquitous nature of *P. fluorescens* on the surface of plants typically grown for human consumption suggests that *P. fluorescens* has been widely consumed by humans for many years. As *P. fluorescens* is not reported to be a causative agent of human food poisoning or other disease related to food ingestion, one can ascertain that *P. fluorescens* is a safe source for production of food grade enzymes.

P. fluorescens is generally considered a nonpathogenic and nontoxigenic species to man (OECD, 1997). The inability of most strains of this species to grow at human body temperature (Palleroni, 1992) limits the organism's invasion and disease-producing capabilities. *P. fluorescens* has been shown to be an opportunistic pathogen in patients who are severely immunocompromised, but is of little concern to immunocompetent individuals (Chamberland *et al.*, 1992; OECD, 1997). Fluorescent pseudomonads have not been reported to be potent allergens; however, they do possess a lipopolysaccharide that may cause an allergic response in some individuals (OECD, 1997).

2.a Literature

An extensive search of the literature was conducted to assess any known potential for *Pseudomonas fluorescens* Biovar 1 strains to be toxigenic or pathogenic to humans. Three separate literature searches were conducted: two searches focused on the toxigenicity and pathogenicity of *P. fluorescens* while the third search was modified to include examination of toxin production by any *Pseudomonas* species. The literature

searches retrieved approximately 800 references. A summary of the results of the literature search is provided in Appendix 14.

2.b Pathogenicity/toxigenicity

As discussed above, a review of the literature shows that *P. fluorescens* is generally considered to be nonpathogenic and nontoxigenic to humans. Several published studies evaluated the pathogenicity and toxigenicity of *P. fluorescens* in mice; no evidence of pathogenicity or toxigenicity was seen under the conditions of the test (George *et al.*, 2000; George *et al.*, 1999).

In addition, the *P. fluorescens* recipient strain MB101 was evaluated in mice (see Section III.10.a.ii and Appendices 10 and 11) and was found to be nonpathogenic and nontoxigenic under the conditions of the test. These findings indicate that *P. fluorescens* is not an infectious threat to humans and is a safe source for production of enzymes intended for use in foods.

2.c Inserted genetic material

In their decision tree for evaluating microbial enzyme safety, Pariza and Johnson (2001) ask several questions relating to the introduced DNA of the genetically modified production microorganism. The first question asks if the expressed enzyme product(s), which are encoded by the introduced DNA, have a history of safe use. Although alpha-amylases themselves have a long history of safe use in food (see Section IV.1.b), the alpha-amylase of this GRAS Notification does not.

The decision tree then questions if the no observable adverse effect level (NOAEL) for the test article in appropriate short-term studies is sufficiently high to ensure safety. Results of the safety studies provided in Section III.10.b, along with the dietary exposure calculations in Section IV.3.b, verify that the NOAEL is sufficiently high to ensure safety.

The next question addresses whether or not the test article is free of transferable antibiotic resistance gene DNA. As outlined in Section II.3, the manufacturing process is such that the alpha-amylase preparation will not contain functional copies of the antibiotic resistance genes.

The decision tree further asks whether all other introduced DNA is well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products. The introduced DNA, plasmids pMYC5088 and pCN51lacI, have been sequenced and gene products are known. The two plasmids were analyzed with computational tools to investigate if adverse proteins were being produced. Both plasmids were found to lack genes that appear to encode known bacterial toxin proteins, virulence factors, or proteins that regulate the expression of bacterial toxin proteins or virulence factors. In addition, published sequence data (of precursor

plasmids), allergic potential assessment results, and toxicity study results all demonstrate that the introduced DNA is free of attributes that would render it unsafe for the proposed uses.

The final question of the decision tree relevant to the genetic modification is whether or not the introduced genetic material is randomly integrated into the chromosome. The two plasmids introduced into strain DC88 are self-replicating, extrachromosomal plasmids thus are *not* integrated into the chromosome; therefore, the question of pleiotropic effects does not apply.

The answers to the above questions are appropriate such that there are no safety concerns regarding the introduced DNA and one can proceed through the decision tree.

3. Enzyme Product

3.a Safety studies

The safety studies outlined in Section III and summarized in Appendix 10 show that the BD5088 alpha-amylase enzyme preparation is safe under its intended conditions of use. The no-observed-adverse-effect-level (NOAEL) found in the 90-day oral toxicity study in rats is **890 mg/kg body weight**, which is roughly 44,500,000 times higher than reasonably anticipated dietary exposure in humans.

3.b Potential dietary exposure

3.b.i Starch

As presented in Section III.9.d above, a calculation based upon actual analysis of glucose processed using BD5088, with none detected at a limit of detection of 2.4 ppb, yields an intake estimate of 1.2 micrograms TOS per person per day. This exposure is 44,500,000 times lower than the NOAEL from the 90-day rat study, also based on TOS. In the case of sweeteners derived from starch, because they are widely distributed in food, *per capita* intake (104 g/person/day) probably approximates mean intake among "eaters only." However, even if the dietary exposure estimate derived from *per capita* sweetener intake is multiplied by a factor of three, there is clearly a very large safety factor (>14,800,000) between the NOAEL and the EDI.

Even if we were to assume—unrealistically—no removal of BD5088 during purification of starch-derived sweeteners, the resulting exposure, 5 mg/person/day, is more than 11,400 times below the NOAEL from the 90-day rat study.

3.b.ii Distilled alcoholic beverages and ethanol for food uses

Any residues of BD5088 α-amylase in distilled alcoholic beverages and ethanol for food uses will be *de minimis* or non-existent and will make a negligible contribution to dietary exposure. Alpha-amylase is used in the early stages of ethanol production and in the thinning of starch in distilling mashes in the alcohol industry. After fermentation of the sugars to produce ethanol, the ethanol is purified by distillation. Because ethanol is relatively low boiling (79°C), no amount of the enzyme protein is reasonably expected to be transferred into the distillate. Accordingly, we regard dietary exposure to BD5088 associated with ethanol to be essentially zero.

4. Manufacturing process

As described in Section II.3, the BD5088 alpha-amylase is produced in accordance with current Good Manufacturing Practice (cGMP) using generally accepted, published methods for enzyme manufacture and formulation. All raw materials used in the alpha-amylase production are of a suitable purity, and the fermentation is run under pure culture, controlled conditions. The final preparation meets the general and additional requirements for enzyme preparations of *Food Chemicals Codex* and JECFA. The manufacturing process for the *P. fluorescens* alpha-amylase is safe.

V) Expert Panel

V. Scientific Expert Panel

A panel of scientific experts with the appropriate education, training and experience in general food safety, microbiology, food enzymology, molecular biology, and toxicology reviewed the scientific data available on the alpha-amylase preparation from *P. fluorescens* strain DC88 and concluded that the enzyme preparation is GRAS, based on scientific procedures, for its intended use. Specifically, the Panel concluded:

> "Based on our independent and collective critical evaluation of the available pertinent scientific evidence, we conclude that Innovase's BD5088 α-amylase, which is derived from *Pseudomonas fluorescens* Biovar I carrying a optimized α-amylase gene constructed from *Thermococcales sp.*, is manufactured in accordance with cGMPs, meets the relevant food grade specifications, and, based on scientific procedures, is 'Generally Recognized as Safe' (GRAS) for use in food as specified herein."

Appendix 15 provides the full conclusions of the panel. Appendices 16 through 19 contain CVs that describe the qualifications through education, training and experience for each Panel member.

VI) Conclusions

VI. Conclusions

The subject of this GRAS Notification is an alpha-amylase enzyme preparation derived from a genetically modified strain of *Pseudomonas fluorescens* Biovar 1 that contains a recombinant gene for an optimized *Thermococcales* alpha-amylase. The enzyme was selected for commercialization due to its stability and activity at low pH, high temperature, and low calcium concentration.

Innovase LLC has determined the alpha-amylase enzyme preparation to be GRAS, through scientific procedures, for use as a processing aid in the starch industry (for the liquefaction of starch in the production of starch hydrolysis products) and in the alcohol industry (for the hydrolysis of starch in distilling mashes). The enzyme is used at minimum levels necessary to achieve the intended technical effects, *e.g.,* up to a maximum of 500 ppm enzyme preparation (containing 3±1% TOS).

The safety of the enzyme preparation has been evaluated using the safety scheme of Pariza and Johnson (2001) and others (IFBC, 1990; JECFA, 2001; Scientific Committee for Food, 1992). Published and unpublished information is provided which assesses the safety of the following: recipient strain, introduced genetic material, production microorganism, alpha-amylases and their use in food, manufacturing process, final alpha-amylase enzyme preparation, and consumer exposure.

The safety of the production organism is the prime consideration when assessing the probable degree of safety of an enzyme preparation intended for use in food. If the enzyme production organism is nonpathogenic and nontoxigenic, and the enzyme is made according to current GMP, then one can assume the food ingredient made from the production microorganism is safe to consume. Strains of *P. fluorescens* are commonly found on plant surfaces as well as on decaying vegetation, and in soil and water. The ubiquitous nature of *P. fluorescens* on the surface of plants typically grown for human consumption suggests that this organism has been widely consumed by humans for many years. *P. fluorescens* strains have been utilized in recent years in a variety of industrial applications, thus the safe environmental use of members of this species is well documented. The recipient strain, *P. fluorescens* MB101, has a history of safe use as the source microorganism in the production of biological pesticides and was experimentally determined to be non-pathogenic and non-toxigenic via oral administration in mice. This finding was confirmed by Dr. Stanley Falkow after examination of the experimental data (see Appendix 11). The production strain DC88 complies with the OECD criteria for Good Industrial Large Scale Practice microorganisms.

The introduced DNA is well characterized and shown to be safe. The DNA sequences of both plasmids are known and an exhaustive open reading frame analysis was done on both plasmids which showed that the two plasmids lack genes encoding known bacterial toxins or virulence factors, and that no dormant or silent genes were introduced during plasmid construction. The toxicity studies performed using alpha-amylase test product further show the introduced DNA is free of attributes that would render it unsafe for use in the production of a food enzyme. The introduced plasmids are non-mobilizable and are

stable in the production strain during storage and fermentation. The food grade alpha-amylase preparations will not contain functional copies of the antibiotic resistance-coding genes.

The enzyme alpha-amylase has a long history of safe use in food processing. Published literature provides evidence that alpha-amylases have been used in food for at least three-quarters of a century. Many GRAS thermostable alpha-amylases are commercially available, several of which are protein engineered. The alpha-amylase from *P. fluorescens* DC88, which is a hybrid of three *Thermococcales* spp. alpha-amylases, is substantially equivalent to other known alpha-amylases in that it contains the four highly conserved regions that are common to all alpha-amylases and is similar at the amino acid level to known amylases. In addition, the BD5088 alpha-amylase is functionally equivalent to other alpha-amylases used in food processing; *i.e.*, it specifically catalyzes the hydrolysis of 1,4-α-glucosidic linkages in amylose and amylopectin.

In assessing the safety of the alpha-amylase preparation, Innovase conducted the following safety studies: 5-day oral gavage in rats; 14-day range finding gavage in rats; 90-day subchronic gavage in rats; 14-day dietary feeding study in rats; mouse lymphoma forward mutation assay; mouse bone marrow micronucleus test; chromosomal aberration assay using rat lymphocytes; and Ames test. The studies did not find any treatment related toxicity or induction of gene mutation or chromosomal aberrations in tests using the alpha-amylase test preparations derived from the production microorganism. Safety margin calculations indicate the potential human exposure to the alpha-amylase enzyme preparation from the consumption of food made using the enzyme is negligible and well below the NOAEL seen in the oral toxicity studies.

The manufacturing process used to make alpha-amylase employs a pure culture submerged fermentation of the *P. fluorescens* DC88 strain. Good Manufacturing Practice is used throughout the process which utilizes generally accepted, published methods for enzyme manufacture and formulation. All raw materials used in the fermentation and recovery processes are of a suitable purity and are standard materials used in the enzyme industry. The final alpha-amylase enzyme preparation meets the general and additional requirements for enzyme preparations as outlined in *Food Chemicals Codex* and by JECFA.

A panel of scientific experts was assembled to review the safety information compiled on the alpha-amylase preparation from the genetically modified *P. fluorescens* source. The panel concluded that the enzyme preparation is GRAS for its intended conditions of use.

Based on the information provided in this GRAS Notification, Innovase concludes that the alpha-amylase preparation derived from *P. fluorescens*, carrying a gene constructed from an optimized *Thermococcales* sp. alpha-amylase, is GRAS under the intended conditions of use, as specified herein.

VII) References

VII. References

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VIII) Appendices

VIII. Table of Appendices

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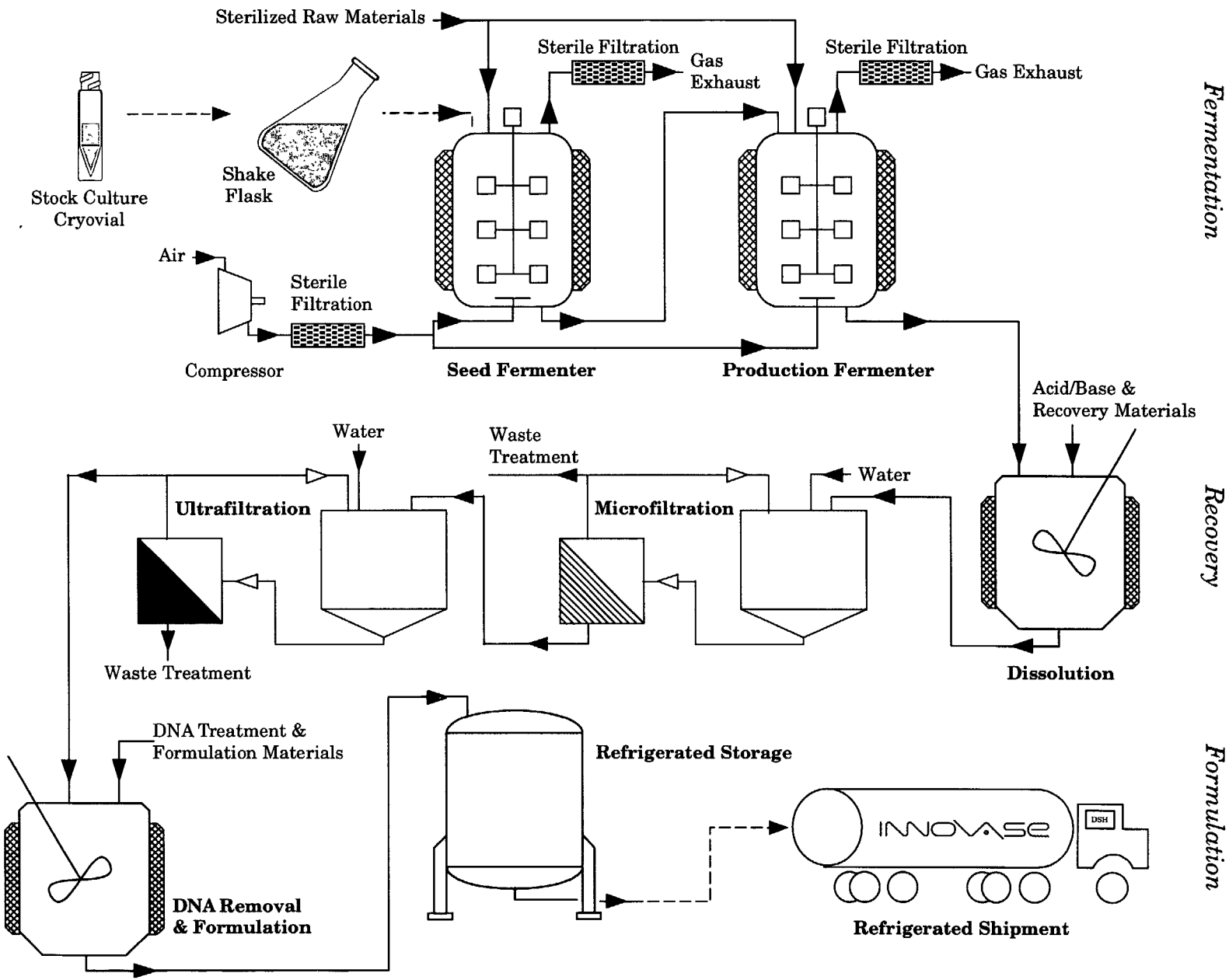
I. Manufacturing

Appendix 1

Manufacturing Process Flow Diagram



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2. Specifications

Appendix 2

PRODUCT SPECIFICATION SHEET

PRODUCT **BD5088 ALPHA-AMYLASE**

SPECIFICATION NO. 1.1
Effective January 21, 2003

Description

Enzyme	Alpha-Amylase (α-amylase)
CAS / EINECS No.	9000-90-2 / 232-565-6
Appearance	Amber liquid
Diluent	Glycerol
pH	10. (typical)
Specific Gravity	1.09 (typical)

Enzyme, Microbiological and Other Assays

Alpha-Amylase Activity	115 IAU per gram – upper limit 100 IAU per gram – lower limit
Total Viable Count	not more than 50000 CFU per gram
Total Coliforms	not more than 30 per gram
E. coli	negative by test in 25 grams
Salmonella	negative by test in 25 grams
Production Organism	negative by test
Antibiotic Activity	negative by test
Lead (Pb)	not more than 5 mg per kg
Heavy metals (as Pb)	not more than 30 mg per kg

Regulatory Compliance

This product complies with the Food Chemicals Codex (FCC 4th ed., 1996) and the JECFA Compendium of Food Additive Specifications (FAO/WHO Vol. 52, Add. 9, 2001) for food grade enzymes, and it contains permitted levels of stabilizers and preservatives. All packaging materials comply with relevant food contact regulations.

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A Joint Venture of The Dow Chemical Company and Diversa Corporation

Innovase LLC
5501 Oberlin Drive
San Diego, CA 92121
U.S.A.

Tel. (858) 352-4454
Fax (858) 352-4560

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

3. Analytical Method

Appendix 3

ANALYTICAL METHOD

Determination of α-Amylase Enzyme Activity in a BD5088 Formulation: 4-Nitrophenyl-α-D-Hexa-Glucopyranoside as Substrate

1. Scope

This method is applicable to measuring enzyme activity of α-amylase in amylase formulation samples and is expressed in Innovase Activity Units (IAU) per gram. This method is also applicable to thermally stable amylases with measurable activity at pH 7.0 and 75°C.

2. Principle

The enzymatic hydrolysis of 4-nitrophenyl-α-D-hexa-glucopyranoside by α-amylase liberates 4-nitrophenol. This liberation can be followed spectrophotometrically at 405 nm. The increase in absorbance at 405 nm is converted to μmoles of 4-nitrophenol by measuring the absorbance at 405 nm of a calibration standard of 4-nitrophenol under the same experimental conditions. One IAU is defined as the quantity of enzyme required to liberate one μmole of 4-nitrophenol from 5 mM 4-nitrophenyl-α-D-hexa-glucopyranoside over one minute at pH 7.0 and 75°C.

3. Safety

Each analyst should be acquainted with the potential hazards of the equipment, reagents, products, solvents and procedures before beginning laboratory work. SOURCES OF INFORMATION INCLUDE: OPERATION MANUALS, MATERIAL SAFETY DATA SHEETS, LITERATURE AND OTHER RELATED DATA. Safety information for non-INNOVASE® products should be released from the supplier. Disposal of waste materials, reagents, reactants, and solvents must be in compliance with laws and regulations from all applicable governmental agencies.

4. Interferences

Any compound that will hydrolyze or liberate 4-nitrophenol from the 4-nitrophenyl-α-D-hexa-glucopyranoside substrate will interfere with this procedure.

5. Apparatus

5.1 Shimadzu UV-1601 and UVPC Software Version 3.9 available from Shimadzu Instruments Inc., 7102 Riverwood Dr., Columbia, GA 21046 or equivalent (Note 14.1).

5.2 IBM-Compatible Personal Computer (PC), NEC MultiSync 4FGe Monitor and keyboard was used to run and control the UV-1601 spectrometer or equivalent computer.

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5.3 Constant-Temperature cell holder for a Shimadzu UV-1601 spectrophotometer, catalog # 202-30858-04, capable of precisely holding the temperature constant of the cuvette from 30°C to 90°C, available from Shimadzu Scientific Instruments Inc. or equivalent.

5.4 Lauda RMT6 constant temperature circulation bath available from Brinkmann Instruments, One Cantiague Road, Westbury, NY 11590-0207 or equivalent.

5.5 Digital thermometer capable of reading temperature accurately to ± 0.2°C between 20°C to 90°C, available from Control Company, 308 West Edgewood, Friendswood, Texas 77546 or equivalent (Note 14.2).

5.6 Vortex-mixer, Thermolyne, Type 16700 Mixer, available from Fisher Scientific Company L.L.C., 2000 Park Lane, Pittsburgh, PA 1527 or equivalent.

5.7 VWR Vanlab heat block catalog # 13259-005, available from VWR Scientific Products, 1310 Goshen Parkway, West Chester, PA 19380 or equivalent.

5.8 Analytical balance, Mettler, Model AE100, capable of weighing to 0.0001 g and a Mettler, Model PC 4400, capable of weighing to 0.01 g available from Mettler-Toledo Inc., Balances and Instruments, 1900 Polaris Parkway, Columbus, OH 43240-2020 or equivalent.

5.9 pH Meter, Denver Instruments, Model 215 and pH/ATC Electrode model 300729, available from Fisher Scientific Company L.L.C., capable of reading to ± 0.01 pH unit or equivalent.

5.10 Magnetic stirrer and stir bar, catalog # 11-500-49S, available from Fisher Scientific Company L.L.C. or equivalent.

5.11 Eppendorf Pipettor, 1.0 mL and 0.1 mL adjustable, available from Fisher Scientific Company L.L.C., 2000 Park Lane, Pittsburgh, PA 15275 or equivalent (Note 14.3).

5.12 Cole-Parmer Sonicator, Model 8853, available from Fisher Scientific Company L.L.C or equivalent.

5.13 Semi-micro, cuvette spectrophotometer cell, 1 cm x 1 cm, 1.16 mL volume, quartz cuvettes. Spectrosil quartz cuvettes are available from Starna, catalog # 9/29-Q-10, Starna Cells, Inc., P.O. Box 1919, Atascadero, CA 93423.

5.14 Class A pipettes, 9.0, 10.0 and 20.0 mL glass pipettes, 100-mL volumetric flask and glass vials (5, 7 and 10-dram), available from Fisher Scientific Company L.L.C. or equivalent.

6. Reagents

6.1 3-[N-Morpholino]propanesulfonic acid, sodium salt (MOPS), 99.5% purity, Sigma # M-9381, available from Sigma-Aldrich, P.O. Box 14508, St. Louis, MO 63178.

6.2 4-Nitrophenyl-α-D-hexa-(1→4)-glucopyranoside(4-Nitrophenyl-α-D-maltohexaoside), >98% purity, Fluka catalog # 73681, Fluka Chemical Corporation, 1001 West St. Paul Avenue, Milwaukee, WI 53233.

6.3 4-Nitrophenol (commonly known as p-nitrophenol) Standard, 99+%, Aldrich # 24132-6, available from Sigma-Aldrich (Note 14.4).

6.4 Hydrochloric Acid, Certified 6.0 N, Fisher # LC15370-2, available from Fisher Scientific Company L.L.C.

6.5 Sodium Hydroxide, Certified 1.0 N, Fisher # SS266-1, available from Fisher Scientific Company L.L.C.

6.6 Buffer Solution pH 7, Certified 7.00 ± 0.01 pH @ 25°C, available from Fisher Scientific Company L.L.C. or equivalent.

6.7 Urea, Certified ACS Grade, 100% purity, Fisher catalog # U15-500, available from Fisher Scientific Company L.L.C.

6.8 Water, 18 mΩ or better, deionized and purified: pass deionized water through a Milli-Q Water System, available from Millipore Corporation Lab Water Division, 80 Ashby Road, Bedford, MA 01730 or equivalent.

7. Preparation of Reagent Solutions

7.1 0.050 M MOPS Working Buffer, pH adjusted to 7.00 ± 0.02 @ ~25°C

7.1.1 Using an analytical balance, weigh between 11.45 and 11.65 g of MOPS (6.1) into a tared, 1000-mL bottle.

7.1.2 Dissolve the MOPS in 900 mL of Milli-Q water (6.8) and stir on a magnetic stirrer until the solids are dissolved.

7.1.3 Calibrate the pH meter with pH 7.00 buffer (6.6) making sure that the buffer solution is stirred with a magnetic stirrer as the pH measurement is made (Note 14.5).

7.1.4 Adjust the pH of the 0.05 M MOPS working buffer to pH 7.00 ± 0.02 @ ~25°C by adding 6 N HCl (6.4). During the final stages of adjusting the pH to 7.00 ± 0.02, one can either use 1 N NaOH (6.5) or 6 N HCl (Note 14.6).

7.1.5 Bring the final volume to 1000 mL with Milli-Q water, label the 0.05 M MOPS working buffer solution with your initials, date prepared and the expiration date. The MOPS solution can be stored at room temperature for up to 90 days.

7.2 1 µmole/mL (1 mM) 4-nitrophenol Standard Solution

7.2.1 Using an analytical balance, weigh 0.14 ± 0.002 g accurately of 4-nitrophenol, 99+% purity (6.3) to the nearest 0.1 mg into a tared, 100-mL volumetric flask.

7.2.2 Dilute the 4-nitrophenol standard (7.2.1) in the 100-mL volumetric flask to volume with the 0.05 M MOPS working pH 7.00 buffer (7.1.5) and mix thoroughly. The weight of 4-nitrophenol used to prepare the calibration standard should be corrected for purity. This solution nominally contains 10.0 mM 4-nitrophenol (note 14.7).

7.2.3 Transfer 10.0 mL of the 10.0 mM 4-nitrophenol standard solution (7.2.2) into a clean 100-mL volumetric flask and dilute to volume with 0.05 M MOPS working pH 7.00 buffer (7.1.5) and mix thoroughly. This solution nominally contains 1.0 mM 4-nitrophenol.

7.2.4 The 4-nitrophenol standard solutions (1.0 mM and 10.0 mM) can be stored at room temperature for up to 5 months. The 4-nitrophenol standard solutions should be stored in either clear or amber glass bottles.

7.2.5 Label the 4-nitrophenol standard solutions with your initials, concentration level, date prepared and expiration date. An expiration date of five months is adequate.

7.3 5 mM 4-Nitrophenyl-α-D-hexa-Glucopyranoside in 0.05 M MOPS Working Buffer Solution

7.3.1 Using an analytical balance, prepare a 5 mM 4-nitrophenyl-α-D-hexa-glucopyranoside (p-NP substrate) solution containing 5.55 mg/mL by weighing between 55 to 230 mg of 4-nitrophenyl-α-D-hexa-glucopyranoside (6.2) to the nearest 0.1 mg into a tared, 50-mL conical centrifuge tube (Note 14.8). Dissolve the p-NP substrate in an appropriate volume of 0.05 M MOPS working pH 7.00 ± 0.02 buffer (7.1.5) such that a concentration of 5.55 mg/mL 4-nitrophenyl-α-D-hexa-glucopyranoside is obtained and mix the solution thoroughly for several minutes (Note 14.9).

7.3.2 Label the p-NP substrate in MOPS working buffer solution with your initials, date prepared and expiration date. The p-NP substrate in MOPS solution should be stored at 4°C, and if the solution turns yellowish prior to the expiration date, it should be discarded and the p-NP substrate solution prepared again (Note 14.10). An expiration date of 4 days or less is recommended.

7.4 6 M Urea Solution

7.4.1 Using a top loading analytical balance, prepare a 6 M urea solution by weighing 35.5 to 36.5 g of urea (6.7) to the nearest 0.1 g into a tared, 100.0-mL volumetric flask, and dissolve the urea in 90.0 mL of Milli-Q water (6.8). Another alternative is to weigh between 355 to 365 g of urea to the nearest 0.2 g into a tared, 1000-mL beaker and dissolve the urea in 900 mL of Milli-Q water.

7.4.2 Slowly stir the urea solution on a magnetic stirrer until dissolved and then adjust the volume to 100-mL or 1.0 L by adding Milli-Q water. Sonication may be used to increase the rate of urea dissolution if needed.

7.4.3 Pour the 6 M urea solution into an appropriate bottle and label the bottle containing the urea solution with your initials, date prepared and expiration date. The 6 M urea solution should be stored at 4°C and can be used for 90 days.

8. Sample Preparation

8.1 The amylase formulation sample is mixed thoroughly for 30 seconds by inverting and shaking the sample several times to homogenize prior to weighing out subsamples for analysis.

8.2 Tare a 5-dram glass vial on an analytical balance. Using a 1.0 mL Eppendorf pipettor, transfer 1.0 mL of formulation sample (1.07 to 1.10 g) into the vial and accurately weigh the amount taken. Add 9.0-mL of 6 M urea solution using a 9.0-mL Class A pipette and mix thoroughly.

8.3 Studies have demonstrated that incubating amylase samples in urea solutions for 5 minutes, 2 hours at room temperature and at 4°C prior to measuring enzyme activity yield equivalent activity results.

8.4 After the formulation sample has been solubilized in the urea solution and thoroughly mixed (8.2 to 8.3), dilute the sample 1:10 with 0.05 M MOPS by taking a 1.0-mL aliquot of the sample solution with an Eppendorf pipettor and diluting with 9.0 mL of MOPS buffer solution. After the samples have been diluted in the MOPS buffer solutions and mixed thoroughly, the sample solution is ready for measuring enzyme activity (Note 14.11).

9. Standardization of the UV/VIS Spectrophotometer

9.1 Set up the Shimadzu UV-1601 spectrophotometer

9.1.1 Turn on both the visible and ultraviolet lamps.

9.1.2 Make sure the Shimadzu UV-1601 spectrophotometer is under computer control.

9.1.3 From the ACQUIRE MODE menu select the TIME COURSE option.

9.1.4 Under the CONFIGURE menu option select the PARAMETERS option and set the Measuring mode: Abs; Recording range: -0.1 to 1.0; Wavelength : 405 nm; Timing mode: MANUAL; Reaction time: 60 seconds; Acquisition rate: 0.1; Number of cells = 1; Interval time = 0.5; and Number of readings: 121.

9.2 Turn on the circulating bath and heating block and make sure both instruments are set to control the temperature at 75°C ± 0.2°C. Each of the holes in the heating block is filled with deionized water to preheat cuvette cells prior to placing them into the Shimadzu spectrophotometer.

9.3 Determine the absorbance for the 1 mM 4-nitrophenol standard

9.3.1 Add 950 µL of 0.05M MOPS buffer solution to the micro cuvette cell and place the cuvette into the heating block set at 75°C for several minutes (Note 14.12).

9.3.2 Remove the cuvette from the heating block, wipe off the water, invert the cell several times and place the cuvette into the reference compartment of the dual beam spectrophotometer.

9.3.3 Repeat this operation of adding 950 µL of 0.05 M MOPS buffer solution into the micro cuvette cell, place the cell into heating block for several minutes, wipe off the water, invert the cell several times and place the cell into the sample compartment of the spectrophotometer. Allow the cuvette cell and contents to equilibrate to 75°C ± 1.0°C for ~3-7 minutes.

9.3.4 During the equilibration process, lightly tap on the cuvette to eliminate air bubbles or remove the cuvette and invert the cuvette several times to release trapped air bubbles.

9.3.5 The absorbance background of the sample cell at 405 nm is nulled by depressing the **AUTO ZERO** key which takes about 3 to 6 seconds to obtain an absorbance reading of 0.000.

9.3.6 Add 50 µL of the 1 mM 4-nitrophenol standard solution to the sample cell cuvette, rinse the contents of the pipettor by gently pipetting the 4-nitrophenol solution up and down several times in the cuvette to mix the solution thoroughly and invert the cuvette two times before placing it into the sample compartment (Note 14.13).

9.3.7 Allow 15-30 seconds for the 4-nitrophenol standard solution to equilibrate to 75°C and record the absorbance of the standard solution. Wait approximately 15 seconds, record the absorbance of the 4-nitrophenol standard solution and then record the absorbance two more times.

9.3.8 If the differences in the first and third absorbance measurements are greater than 0.01 AUFS, measure the absorbance of the 4-nitrophenol standard a fourth time.

9.3.9 Record the three absorbance values for the 1 mM 4-nitrophenol standard and calculate an average absorbance value as the final result. Typically, an absorbance value from 0.36 to 0.40 AUFS is obtained for the 1 mM 4-nitrophenol calibration standard (Note 14.14).

9.3.10 Repeat steps 9.3.1 to 9.3.9 for at least one or two additional 4-nitrophenol standardizations and average the standardization results. The standardization procedure is generally carried out in triplicate before and after the enzyme activity of the amylase sample solutions are measured (Note 14.15).

10. Determination of α-Amylase Activity

10.1 Add 950 µL of 5 mM 4-nitrophenyl-α-D-hexa-glucopyranoside (p-NP substrate) in the 0.05 M MOPS working buffer solution (7.3.1) into a micro cuvette cell and place the cuvette into the heating block set at 75°C for several minutes.

10.2 Remove the cuvette from the heating block, wipe off the water, invert the cell several times and place the cuvette into the sample compartment of the dual beam spectrophotometer. Allow ~3 to 6 minutes for the cell to equilibrate to 75° ± 1.0°C.

10.3 Add 50 µL of the 1:10 dilution of the α-amylase sample solution (section 8.4) to the cuvette, mix well by gently pipetting up and down several times and invert the cell two times (the mixing operation usually takes less than 15 seconds).

10.4 Blank the absorbance of the sample cell at 405 nm by depressing the **CELL BLANK** key.

10.5 Once the **CELL BLANK** key is depressed, quickly depress the **START** key to initiate the measurement of absorbance versus time at 405 nm for 60 seconds.

10.6 An increasing linear absorbance should be seen on the spectrophotometer screen or monitor. At the end of 60 seconds, the computer will ask for you to enter a **FILE NAME** (i.e. DATE-01, DATE-02, DATE-03...) for the amylase sample solution. Enter a file name and save the file.

10.7 Under the **MANIPULATE** menu option, select the **DATA PRINT** option. Under the **DATA PRINT** option, select the Act. Cal. Option and with a Multiplication factor of 60 (60 seconds/minute) the activity measurement (dA/minute result) is calculated and shown. The activity result is recorded (Note 14.16).

10.8 Repeat steps 10.1 to 10.7 twice for each amylase standard solution and average the results. Occasionally, the two activity results will differ by 3% or more and a third activity measurement is made. In these cases, the three activity measurements are averaged together to obtain the final result.

11. Calculations

11.1 Calculate the urea dilution factor for urea dissolved samples as follows (Note 14.17):

$$\text{Urea Dilution Factor} = \frac{\text{(Volume of Urea [mL] + [Sample Amount (g)/1.09 g/mL])}}{\text{[Sample Amount (g)/1.09 g/mL]}}$$

11.2 Calculate the sample dilution factor for diluting the α-amylase sample in urea with 0.05 M MOPS solution as follows (Note 14.18):

$$\text{Sample Dilution Factor} = \frac{\text{(Volume of Urea Diluted Sample Solution + Volume of MOPS Solution)}}{\text{Volume of Urea Diluted Sample Solution}}$$

11.3 Calculate the final dilution factor as follows:

Final Dilution Factor = (Sample Dilution Factor) x (Urea Dilution Factor)

11.4 Calculate the activity of the α-amylase enzyme sample (IAU/g) as follows (Note 14.19):

$$\text{Activity (IAU/g)} = \frac{\text{(Final Dilution Factor) x (dA/min measurement for sample)}}{\text{(sample wt.) x Average absorbance of 1 mM 4-nitrophenol standard}}$$

12. Precision

A precision study was carried out by preparing five 1.0 mL subsamples of an α-amylase formulation sample as described in Section 8. Each of the 1/10 dilution of the samples in 0.05 M MOPS were analyzed for enzyme activity once each day on three successive days. A 4-nitrophenol calibration standard was also analyzed at least six times, the calibrations were averaged together and used to calculate the final activity result. Based on the activity results obtained on the fifteen sample analyses, standard deviation and relative standard deviation (% RSD) were calculated. These values may be expected to vary from the average by not more than the amounts listed (relative standard deviation).

Component	Concentration	Activity (Units)	Standard Deviation	RSD	Precision
Amylase activity	10 g/L	113.1	2.187	1.93%	4.15 %

13. Linearity

A linearity study was conducted by measuring the absorbance at 405 nm of seven different 4-nitrophenol concentrations at 75°C. The 4-nitrophenol standards ranged from 0.125 mM to 2.5 mM in concentration and were analyzed according to the procedure described in Section 9. A

graph of absorbance versus 4-nitrophenol concentration is shown in Figure 1. A summary of the linearity data is shown below in Table I.

Table I
Absorbance at 405 nm for a
Series of 4-Nitrophenol Standards

4-Nitrophenol Concentration	Absorbance @ 405 nm
0.125 mM	0.0475
0.250 mM	0.0945
0.500 mM	0.1835
0.750 mM	0.2715
1.000 mM	0.3640
1.250 mM	0.4530
2.500 mM	0.8965

Linearity Curve Equation is $y = 0.3572*X + 0.0047$ with $R^2 = 1.000$

14. Notes

14.1 The spectrophotometer must have the capability to measure the change in absorbance versus time and express the result as the average absorbance rate change over one minute (dA/min). Some spectrophotometers have software for converting the absorbance versus time curve into a direct dA/min result. Other spectrometers do not have this feature. The Shimadzu Model UV-1601 reports the actual dA/min result.

14.2 The digital thermometer used to monitor the heating/circulating bath and spectrophotometer cell temperature should be calibrated against a NIST-traceable standard so that the temperature measured is accurate to 0.2°C.

14.3 The Eppendorf pipettor should be calibrated prior to use in this procedure. A common method of calibrating the pipettor is to transfer 100 µL volumes of water into a tared vial placed on an analytical balance and weigh the amount (mg) of water pipetted. By repeating this procedure several times, the average amount of water per 100 µL aliquot (mg/µL) can be calculated and used to determine the exact volume transferred with the pipettor.

14.4 A primary standard of 4-nitrophenol should be obtained and characterized for purity or a "Certificate of Analysis" obtained from the vendor that confirms the purity prior to use. The purity of 4-nitrophenol standard can be established by, differential scanning calorimetry, base titrimetry, liquid and gas chromatographic analysis.

14.5 The absorbance of the 4-nitrophenol standard solution is extremely sensitive to slight pH changes (50% absorbance change for a 0.2 pH unit difference near pH 7). Consequently, the pH of the 0.05 M MOPS working buffer must be carefully and precisely adjusted to pH 7.00 and this requires that the calibration buffer and 0.05 M MOPS working buffer solutions are stirred during pH measurements.

14.6 The pH meter must be calibrated just prior to measuring and adjusting the pH of the 0.05 M MOPS working buffer and the pH measurement must be stable for several minutes. After the MOPS buffer solution has been adjusted to pH 7.00, the pH of the pH 7 buffer solution should be measured again to insure that the pH meter reading has not drifted. If the pH meter reading has drifted, the pH of the 7.00 buffer and 0.05 M MOPS working buffer solutions should be measured again.

14.7 The absorbance of several 4-nitrophenol standards prepared in the MOPS buffer @ pH 7.00 were measured to determine the stability of these solutions over time. The 1 mM and 10 mM concentrations of 4-nitrophenol standard prepared in the MOPS buffer @ pH 7.00 are stable for at least 5 months when stored in either clear or amber glass bottles at room temperature.

14.8 Because the 4-nitrophenyl-α-D-hexa-glucopyranoside (p-NP substrate) statically clings to spatulas and other weighing utensils during the weighing process, it was easier to tare a 50-mL conical tube in a 100-mL beaker and add the p-NP substrate reagent while lightly tapping on the side of the container. When the p-NP substrate was added in this manner, it was easy to control and transfer within 2-5 mg of the desired weight of p-NP substrate into the conical tube.

14.9 To minimize day-to-day differences for activity measurements, it was important to prepare the exact concentration (5.55 mg/mL) of p-NP substrate reagent. This means that the p-NP substrate should be weighed accurately and the exact volume of 0.05 M MOPS working buffer was added to prepare a 5.55 mg/mL p-NP substrate concentration. Typically, 111 mg is weighed out and dissolved in 20.0 mL of MOPS working buffer solution.

14.10 To minimize p-NP substrate hydrolysis (yellow color formation), the conical container containing p-NP substrate solution should be stored in an ice bath during activity measurements.

14.11 All sample dilutions must be made in the 0.05 M MOPS buffer using a minimum volume of at least 100-µL of enzyme sample. The analyst is free to scale these dilutions up or down to increase the accuracy and repeatability of these measurements as long as the absorbance falls within the 0.05 to 0.60 AUFS range.

14.12 The 0.05 M MOPS buffer should be kept at ambient temperature prior to transferring to the cuvette. Another alternative to heating the cuvette and MOPS buffer in the spectrophotometer is to preheat the cuvette containing the MOPS buffer in a heating block at 75°C and transferring the heated cell and contents to the spectrophotometer to minimize equilibration time @ 75°C prior to adding the p-NP standard or sample additions.

14.13 If the absorbance of the MOPS buffer solution does not change by more than 0.003 AUFS during the equilibrating period, then the cuvette and contents have attained 75°C. The 0.05 M MOPS solution is not sufficiently stable @ 75°C for extended periods of time. For the best results, cuvettes should be heated for exactly the same length of time during the

preheating and analysis steps of the assay procedure. A digital thermometer can be placed into the cuvette and the temperature should be 75°C ± 1.0°C. To obtain an accurate absorbance measurement, no bubbles should be visible in the cuvette.

14.14 The absorbance reading should be constant or within ± 0.002 AUFS. If the measured absorbance is continuously dropping, the cell and contents are not at constant temperature. Do not record the absorbance measurement until constant temperature is reached.

14.15 Typically, the absorbance for 1 mM 4-nitrophenol standard should range from 0.36 to 0.40 AUFS. If the absorbance difference between two 1 mM 4-nitrophenol standard preparations is greater than 0.006 AUFS, then a third absorbance measurement for 1 mM 4-nitrophenol should be made and all standardizations averaged together.

14.16 The Cary 100, Beckman DU-7400 and Shimadzu UV-1601 spectrometers all give a direct absorbance versus time measurements of dA/second or dA/min.

14.17 Since the density of the amylase formulation samples is equivalent to 1.09 g/mL, a 1.00 mL sample is equivalent to 1.09 g. If the analyst follows the sample preparation instructions as described in Section 8.1 to 8.4 for α-amylase samples, the Urea Dilution Factor is ~10.0.

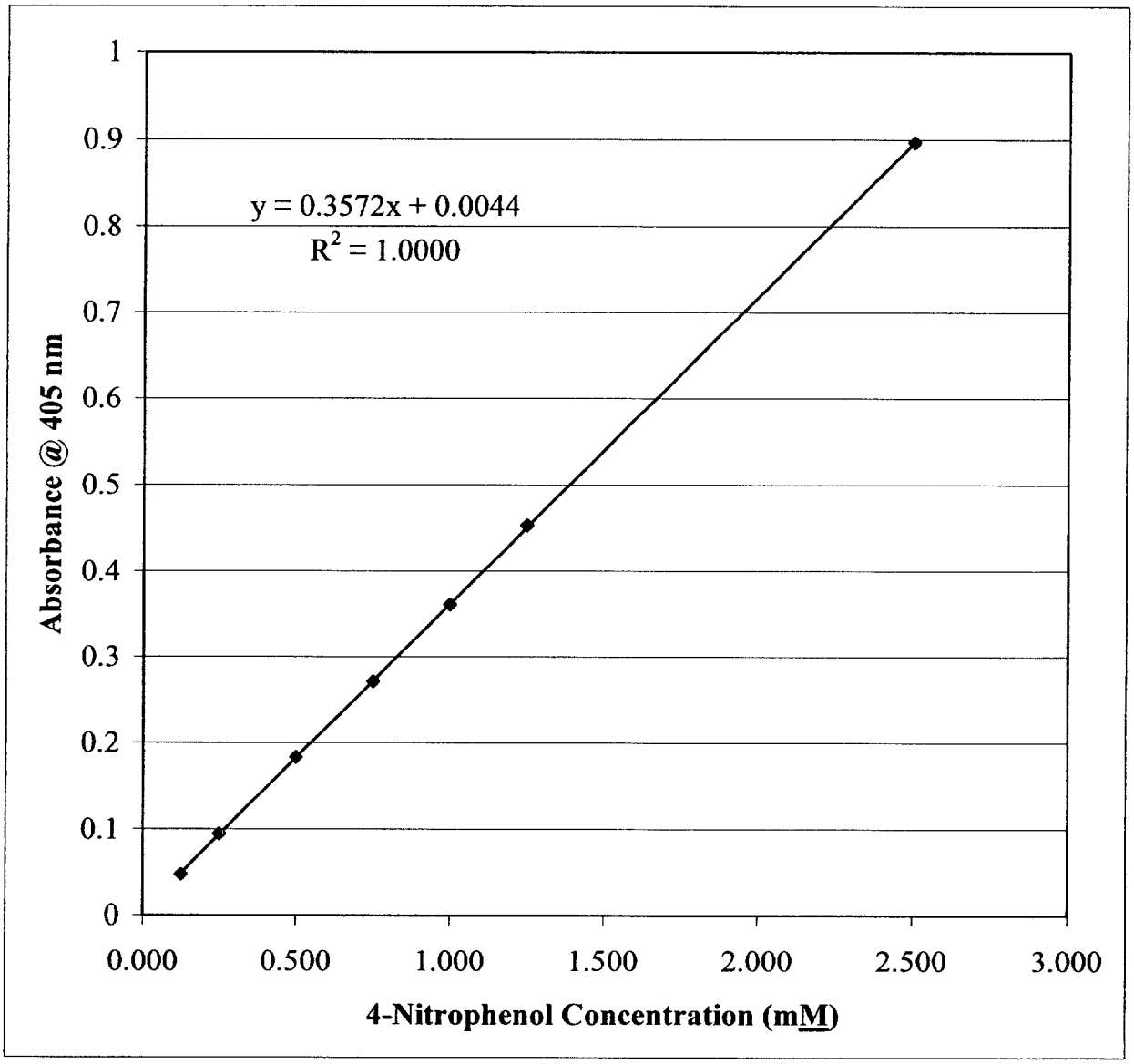
14.18 If the analyst follows the sample preparation instructions as described in 8.4 for formulation samples of α-amylase, the Sample Dilution Factor is 10 for diluting the sample 1 to 10.

14.19 If the analyst follows the sample preparation instructions as described in Section 8 for formulation samples of α-amylase, the Final Dilution Factor is ~100. The sample weight is also used in calculating the final activity results in U/g.

Figure 1

Analytical Method Linearity Study

Absorbance at 405 nm as a Function of 4-Nitrophenol Concentration



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4. Strain ID

Appendix 4

June 15, 1988 letter from Prof. I. J. Misaghi, Department of Plant Pathology, University of Arizona, verifying recipient strain MB101 as *Pseudomonas fluorescens* biotype A. Biotype A is equivalent to Biovar I (see Appendix 5, Table 3).

6-15-88

Dr. Gregory Gibb
Mycogen Corp.
5451 Oberlin Dr.
San Diego CA 92121

I.J. Misaghi
Department of plant pathology
Universisty of Arizona
Tucson, AZ 85721

Dear Greg,

Judging from the results of a number of diagnostic tests, the culture designated as MB101 is Pseudomonas fluorescens. With respect to biotype designation, it is close to biotype A of P. fluorescens(ATCC 17397).

The culture is oxidase positive, gram negative, hypersensitive negative on tobacco, pectolytic negative on sodium polygalacturonate, with API Rapid NFT seven diget profile of 0147555.

Please feel free to call(602 621 7156) or to write if you have any questions.

Sincerely,

(b)(6)

I. J. Misaghi
Associate Professor

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Reactions of MB101 to API Rapid NFT Tests.

		Reaction
Nitrate reduction	[Production of NO_2	-
	[Production of N_2	-
Tryptophanase		-
Glucose Fermentation		-
Presence of arginine dihydrolase		+
Presence of urease		-
Hydrolysis of esculin		-
Presence of gelatinase		-
Presence of Beta-galactosidase		-
Assimilation of D-glucose		+
Assimilation of L-arabinose		+
Assimilation of D-Mannose		+
Assimilation of D-Mannitol		+
Assimilation of N-Acetyl-D-glucosamine		+
Assimilation of Maltose		-
Assimilation of D-Gluconate		+
Assimilation of Caprate		+
Assimilation of Adipate		-
Assimilation of Malate		+
Assimilation of Citrate		+
Assimilation of Phenylacetate		-
Presence of oxidase		+

Received from
D. I. Msegh
on 7/14/88

000072

5. Strain Characterization

Appendix 5

Characterization and Identification of the Recipient Strain MB101 as *Pseudomonas fluorescens* Biovar I

INTRODUCTION

Microbial enzymes used in food processing are typically sold as enzyme preparations that contain not only the desired enzyme activity but also other metabolites of the production strain, as well as added formulation materials such as preservatives and stabilizers. As outlined by Pariza and Johnson (2001), the safety of the production strain is the primary consideration in evaluating enzyme safety. The critical elements required to establish a safe production strain include thoroughly characterizing the recipient organism, determining the safety of all new DNA that has been introduced into the recipient organism, and ensuring that the procedure(s) that have been used to modify the recipient organism are appropriate for food use. In this report, the recipient and production strains (MB101 and DC88, respectively) are characterized. Both phenotypic and genotypic analyses were performed to establish the taxonomy of strain MB101. Phenotypic characterization included a number of biochemical tests including development of whole-cell fatty acid methyl ester (FAME) profiles, whereas genotypic characterization encompassed 16S rRNA analysis. The results show that strains MB101 and DC88 are both *P. fluorescens* Biovar I strains.

MATERIALS AND METHODS

Bacterial Strains

Bacterial strains were originally obtained from the American Type Culture Collection (ATCC). Table 1 shows the bacterial strains used in these studies.

Table 1. Bacterial strains employed in this study.

Organism Name	*Pseudomonas* Species	Biovar	Alternate Designation
MB101	*P. fluorescens*	Biovar I	Recipient strain. Identity verified herein.
MB214	*P. fluorescens*	Biovar I	MB101 derived. Identity verified herein.
DC88	*P. fluorescens*	Biovar I	Production strain. Identity verified herein.
LMG5825	*P. fluorescens*	Biovar I	ATCC 17813
LMG5830	*P. fluorescens*	Biovar I	ATCC 17556
LMG5916	*P. fluorescens*	Biovar I	ATCC 17397
MB295	*P. fluorescens*	Biovar I	ATCC 13525; DSM50090
MB237	*P. fluorescens*	Biovar I	ATCC 17397
LMG1244	*P. fluorescens*	Biovar III	ATCC 17571
LMG5822	*P. fluorescens*	Biovar III	ATCC 11251
LMG5831	*P. fluorescens*	Biovar III	ATCC 17581
LMG5938	*P. fluorescens*	Biovar III	ATCC 17559
LMG5939	*P. fluorescens*	Biovar IV	ATCC 12983
LMG5167	*P. fluorescens*	Biovar V	ATCC 17386
LMG5833	*P. fluorescens*	Biovar V	ATCC 17518
LMG5940	*P. fluorescens*	Biovar V	ATCC 17562
MB285	*P. fluorescens*	ND	NRRL B-4290R (R = rough)

MB296	*P. aeruginosa*	NA	ATCC 10145; Type strain
MB298	*P. aureofaciens*	NA	ATCC 13985; Type strain
DSM6698	*P. aureofaciens*	NA	DSM 6698T; Type strain
MB297	*P. chlororaphis*	NA	ATCC 9446; Type strain
LMG5004	*P. chlororaphis*	NA	ATCC 9446; Type strain
LMG2210	*P. marginalis*	NA	ATCC 10844; Type and pathovar reference strain
MB184	*P. putida*	ND	NRRL B-1595
MB185	*P. putida*	ND	NRRL B-1635
MB294	*P. putida*	Biovar A	ATCC 12633
LMG2342	*P. tolaasii*	NA	ATCC 33618; Type strain
LMG2352	*P. viridiflava*	NA	ATCC 13223; Type strain

(ND: not determined. NA: not applicable.)

Levan Production

The pseudomonads were grown on Nutrient Agar supplemented with 5% sucrose where levan-producing colonies formed white, convex mucoid colonies (Schaad, 1988; Stanier *et al.*, 1966 with specific reference to levan production on p. 205 and table 12).

Phenizine Production

The pseudomonads were analyzed for the production of pigments on King's A medium (Stanier *et al.* 1966, pp. 172-173; Palleroni 1986, p.158). King's A medium is described in Bergey's Manual (1984).

Lecithinase

The pseudomonads were analyzed for formation of cloudy, opaque zones around colonies after growth on egg yolk agar (Stanier *et al.* 1966, p. 175; Smibert and Krieg 1981, pp. 417-418).

Denitrification

The pseudomonads were grown in media where nitrogen, in lieu of oxygen, is used as the terminal electron acceptor. The mineral base media is described in Palleroni and Doudoroff (1972) and the method and media supplements is described in Stanier *et al.* (1966, p. 171 and 182).

Gelatin Hydrolysis

The pseudomonads were grown in L-broth supplemented with 4% or 12% gelatin. Gelatin hydrolysis was observed after chilling at refrigerator temperatures (Smibert and Krieg 1981, pp. 415-416). 12% gelatin usage yielded more sensitive results.

Trehalose and Adonitol Utilization

Trehalose and adonitol utilization of strain MB101 was determined using the Biolog GN Microplate test system (Biolog Inc., Haywards, CA).

Carbon Utilization

All strains were initially grown on L (Luria)-agar and all tests initiated from YA, as described in Palleroni and Doudoroff (1972) and in Stanier *et al.* (1966). Mineral base media was prepared 2x as was the purified agar. Preparation and concentration of organic

sources was as described in Palleroni and Doudoroff (1972). Two-fold concentrated agar was added to mineral base along with the appropriate amount of the fully sterilized carbon source to yield the final component concentration as outlined in Palleroni and Doudoroff (1972). Each component was tested in duplicates by the replica plating method described in Lederberg and Lederberg (1952). Plates were incubated for 3-4 days at 30°C and then analyzed for growth.

Fatty Acid Methyl Ester (FAME) Analysis

Cell Preparation:

The FAME analysis preparation of the *Pseudomonas* strains was performed as follows:

- *Pseudomonas* strains were grown on King's B agar plates for 16 hrs at 30°C. (King's B agar: 20g proteose peptone #3, 10g glycerol, 1.5g K_2HP0_4, 1.5g $MgS0_4$-$7H_20$, 15g agar; heated until dissolved in 1L deionized water; pH to 7.2; autoclaved 30 min at 121°C).
- Cell mass (40-50 mg) was transferred to a screw-cap test tube with a Teflon-lined screw cap.
- One milliliter of 14% boron trifluoride dissolved in methanol was added and the tube was then shaken to separate the fatty acids from the interference, esterify and methylate them to form volatile methyl esters of the free fatty acids. The mixture was heated 15 min at 100°C in a heat block followed by cooling in tap water. 1 ml saturated NaCl (40g NaCl/100ml deionized water) was added.
- FAMEs were extracted into 1 ml n-hexane then centrifuged at low speed for 1-2 minutes to separate into layers.
- The upper hexane layer was transferred into septum-covered chromatography vials to be analyzed by gas chromatography (GC).

Gas Chromatography:

Gas chromatographic analysis was performed on a Hewlett Packard 5890 instrument connected to a Hewlett Packard 3396A integrator: The inlet system was a split-capillary injector operated at 250°C in the septum-purge mode (purge rate, 0.5ml/min) at a split ratio of 1:50. The column was a 50% cyanopropyl - 50% methyl, 0.25 mm x 30 m fused silica capillary column (DB23, J &W Scientific, Folsum, CA) operated with hydrogen as the carrier gas. Linear flow was 40 cm/sec. The oven temperature was programmed as follows: initial temperature, 100°C; hold time 0 min; heating rate, 5°C/min; final temperature, 200°C; final time, 2 min. The detector was a flame ionization detector operated at 250°C with helium as the capillary make-up gas. Once the system was operational, the auto-injector injected 4µl of the cellular fatty acid extracts. The integrator processed the chromatographic data to yield peaks for each fatty acid at separate retention times, along with peak area and percent peak area.

Data Analysis:

The fatty acid profiles were entered as a matrix that correlates the quantity of a given fatty acid (*e.g.* % peak area at a given retention time) to a particular strain. A statistical package (SYSTAT V. 5.0, from Systat, Inc., Evanston, IL) was used to perform a hierarchical CLUSTER analysis on the matrix to result in a similarity index described as follows from the SYSTAT manual. The program measures dissimilarity between objects.

Similar objects should appear in the same cluster and dissimilar objects in different clusters. The EUCLIDEAN distance measure was used to examine the data. This option uses normalized Euclidean distance, *i.e.* root mean squared distance. The linkage method (amalgamation) used to compute the distance of one object or cluster from another and to determine whether the two should be merged in a given step was SINGLE LINKAGE clustering. This linkage method takes the distance between two objects or clusters as the distance between the two closest members of those clusters and yields a "nearest neighbor" tree diagram. A single subroutine follows the hierarchical amalgamation method. The algorithm in Grurcers and Wainer (1972) is used to order the tree (Wilkenson, 1990).

Establishment of the 16S rRNA Gene Sequences of Strains MB101 and DC88

The PCR amplification was carried out for 30 cycles using the first six primers listed in Table 2 and 100 ng genomic DNA of strain MB101 and DC88 as template. The following temperature profile was used: 60 sec at 95°C for DNA denaturation, 30 sec at 55°C for primer annealing, and 90 sec at 72°C for DNA extension. The DNA sequence was established using the six primers used in the PCR as well as 3 new internal primers for sequencing (HV2F, HV1R, and HV3F; see Table 2). DNA sequencing was performed using the dye-terminator fluorescence sequencing method following the protocol of Applied Biosystems. G50 columns were used to clean up the reactions. The dried DNA was resuspended in formamide and was loaded onto the ABI 3100 DNA Analysis System (Applied Biosystems, Foster City, CA) for sequence determination.

Table 2: Overview of primers used for PCR-amplification, and DNA sequence establishment.

Primer Name	Size	Sequence (5' → 3')	Size of PCR Product
HV1F2	19mer	ATTGAACGCTGGCGGCAGG	574 bp
HV2R	22mer	CACATCCAACTTAACAAACCAC	
HV1F2	19mer	ATTGAACGCTGGCGGCAGG	1132 bp
HV3R2	20mer	CCGGCAGTCTCCTTAGAGTG	
HV1F3	20mer	AGAGTTTGATCCTGGCTCAG	1500 bp
HV3R1	21mer	ACGGCTACCTTGTTACGACTT	
HV2F	21mer	TGAGGTAATGGCTCACCAAGG	NA
HV1R	20mer	ATGGCTGGATCAGGCTTTCG	NA
HV3F	21mer	GCTAACGCATTAAGTTGACCG	NA

Computational Analysis

The raw DNA sequences were assembled using the Sequencher DNA assembly software (Gene Codes Corporation, Ann Arbor, MI). Database searches were performed using the BLASTN function of GCG (Accelrys, San Diego, CA). Binary alignments were done with the GAP program of GCG using the default parameters. Multiple sequence alignments were generated using the GCG program PILEUP with the default parameters.

RESULTS

1. STRAIN HISTORY

The species *Pseudomonas fluorescens* is an abundant, cosmopolitan, and natural component of the microbial flora of soil, water and plants. Members of the species *P. fluorescens* are aerobic, saprophytic, gram-negative organisms that are not pathogenic (do not cause a disease state) in plants, mammals or immunocompetent humans. They grow at neutral pH and in the mesophilic temperature range (optimally between 25 and 30°C), and do not grow above 40°C or under acid conditions (<pH 4.5) (Bergey's Manual, 1984, 1994; OECD 1997).

Pseudomonas fluorescens strain MB101 was isolated in March 1984 from the phytoplane of a young lettuce leaf located on a farm in Northern San Diego County, California Alpha-amylase production strain DC88 is a derivative of MB101 that contains two plasmids, pMYC5088 and pCN51lacI.

No single technique or methodology can positively distinguish *P. fluorescens* from all other potentially unrelated species and Biovars, nor can a single technique prevent inadvertent exclusion of a related species or Biovar. However, the concerted approach described herein which utilizes morphologic, phenotypic and genotypic techniques *is* capable of conclusively identifying *P. fluorescens* down to the Biovar level *and* simultaneously excluding other unrelated species and Biovars.

2. MORPHOLOGIC CHARACTERIZATION

Morphologic characteristics for MB101 and DC88 are consistent with those identified in Bergey's Manual (1984, 1994) for *Pseudomonas fluorescens*:

- Straight or slightly curved rods, but not helical
- Motile by one or several polar flagella
- 0.5-1.0 µm in diameter by 1.5-5.0 µm in length
- Do not produce prosthecae and are not surrounded by sheaths

3. PHENOTYPIC CHARACTERIZATION

Phylogenetic classification of *P. fluorescens* has relied on a series of nutritional characteristics to differentiate seven distinct biotypes (A-G) of *P. fluorescens* from other fluorescent pseudomonads (Stanier *et al.* 1966). Currently, the most recent edition of "Bergey's Manual of Systematic Bacteriology" (1984) recognizes only five Biovars (*i.e.*-biotype) of *P. fluorescens* since *P. fluorescens* biotypes D and E have been reclassified as distinct species (*P. chlororaphis* and *P. aureofaciens*). Over the years Biovar and biotype have been used interchangeably as a designation for the same strain of *P. fluorescens*. The relationship between the Biovar/biotype designation is shown in Table 3.

Table 3: Relationship between biotype and Biovar Designation for *P. fluorescens*.

Biovar as designated by Stanier *et al.* (1966)	Biovar I	Biovar II	Biovar III	Biovar IV	Biovar V
Biotype equivalent	biotype A	biotype B	biotype C	biotype F	biotype G

In order to establish the phylogeny of strain MB101, this organism was independently characterized in terms of its morphological and biochemical properties by Professor I. J. Misaghi of the Department of Plant Pathology at the University of Arizona at Tuscon (Attachment I). The results from a series of phentoypic tests confirmed that strain MB101 belongs to *P. fluorescens* Biotype A, *i.e.*, Biovar I. The tests included API rapid NFT, production of levan and phenizine, presence or absence of lecithinase, ability to denitrify and liquefy gelatin, utilization of trehalose and numerous other carbon sources, and analysis of fatty acid methyl esters. Each of these tests is described in more detail below.

API rapid NFT Test

API rapid NFT tests (Analytab Labs, Plainview, NY) were performed by both Dr. I. J. Misaghi and by company research personnel. This test system is specifically designed for identification of Gram-negative non-fermentative bacteria. Strain MB101 was identified as *P. fluorescens* based on an API code of 0147555 (confidence of 99.8%). A second series of analyses was conducted by company personnel using the identical API rapid NFT test. Both MB101, as well as on a positive control *P. fluorescens* strain ATCC 17397, were identified as *P. fluorescens* based on API codes of 0347555 (confidence of 99.8%). The results from these analyses are presented in Table 4.

Levan Production

Production of levan from sucrose was used to further characterize MB101. *P. fluorescens* Biovars I, II, and IV produce levan while *P. fluorescens* Biovars III and V, *P. putida* and *P. aeruginosa* (Bergey's Manual, 1994) are negative for this phentoypic characteristic. Strain MB101 was shown to produce levan (Table 5).

Phenizine Production

The production of phenizine pigment is used to distinguish between *P. fluorescens* (negative), *P. putida* (negative), and *P. aeruginosa* (positive). Strain MB101 does not produce any of these pigments (Table 5). The two *Pseudomonas* species *P. chlororaphis* and *P. aureofaciens* are shown as controls for other pigments they produce which are different from the phenizine pigment produced by *P. aeruginosa.* These results are in agreement with those presented in Bergey's Manual (1994).

Lecithinase

This test clearly distinguishes *P. fluorescens* MB101 (positive) from the *P. putida* strains and *P. aeruginosa,* both of which are negative (Table 5). These results are in agreement with those presented in Bergey's Manual (1994).

Denitrification

This test clearly distinguishes strain MB101 (negative) from the positive *P. aeruginosa* and *P. chlororaphis* strains (Table 5). These results are in agreement with the information presented in Bergey's Manual (1994).

Table 4: Reactions of Strain MB101 in API Rapid NFT Tests Performed by Professor Misaghi and Confirmatory Testing.

Test	Reaction (Misaghi)	Reaction (Company)
Nitrate Reduction		
Production of NO_2	–	–
Production of N_2	–	–
Tryptophanase	–	–
Glucose Fermentation	–	–
Presence of Arginine Dihydrolase	+	+
Presence of Urease	–	–
Hydrolysis of Esculin	–	–
Presence of Gelatinase	–	–
Presence of β–Galactosidase	–	–
Assimilation of D–Glucose	+	+
Assimilation of L–Arabinose	+	+
Assimilation of D–Mannose	+	+
Assimilation of D–Mannitol	+	+
Assimilation of N–Acetyl–D–Glucosamine	+	+
Assimilation of Maltose	–	–
Assimilation of D–Gluconate	+	+
Assimilation of Caprate	+	+
Assimilation of Adipate	–	–
Assimilation of Malate	+	+
Assimilation of Citrate	+	+
Assimilation of Phenylacetate	–	–
Presence of Oxidase	+	+

Gelatin Hydrolysis

Although Bergey's Manual (1994) shows all *P. fluorescens* Biovars as positive for gelatin hydrolysis, negative results were obtained for MB101 by this method and by the gelatin analysis in the API Rapid NFT system (Tables 4 & 5). Both *P. putida* Biovars are shown as negative, while *P. aeruginosa, P. chlororaphis* and *P. aureofaciens* are all positive.

Since numerous number of strains of *P. fluorescens* have been isolated that do not hydrolyze gelatin, this characteristic is no longer a universal phenotypic trait of *P. fluorescens.* The API identification system supports that finding in that only 55% of *P. fluorescens* strains recorded in their database were reported to hydrolyze gelatin.

Table 5. Biochemical Identification Tests for *P. fluorescens*

Organism Name	*Pseudomonas* Species / Biovar	Levan Production	Phenizine Production	Lecithinase	Denitrification	Gelatin Hydrolysis
MB101	*P. fluorescens* Biovar I (Pf bv I)	+	–	+	–	–
MB214	*P. fluorescens* (Pf)	+	–	+	–	–
MB237	*P. fluorescens*	+	–	+	–	–
MB285	*P. fluorescens*	+	–	+	–	–
MB295	*P. fluorescens* Biovar I	+	–	–	–	–
MB184	*P. putida* (Pp)	–	–	–	–	–
MB185	*P. putida*	–	–	–	–	–
MB294	*P. putida* Biovar A (Pp bv A)	–	–	–	–	–
MB296	*P. aeruginosa* (Pae)	–	+ [1]	–	+ [4]	+
MB297	*P. chlororaphis* (Pc)	+	+ [2]	+	+ [5]	+
MB298	*P. aureofaciens* (Pau)	+	+ [3]	+	–	+

[1]Blue, pyocyanin; [2]Green crystals, chlororaphin; [3]Orange, phenizine a-carboxylic acid; [4]Produces gas; [5]No gas production

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Trehalose and Adonitol Utilization

Trehalose utilization is another of the classical phenotypic characteristics used to distinguish *P. fluorescens* (positive) from *P. putida* (negative). Table 6 shows the results obtained for trehalose and adonitol utilization using the Biolog GN Microplate test system. The ability of MB101 to utilize trehalose clearly differentiates this strain from either *P. aeruginosa* or *P. putida* Biovar A or Biovar B. The positive adonitol utilization distinguishes MB101 from *P. aeruginosa,* both Biovars of *P. putida,* and Biovars II and IV of *P. fluorescens, P. chlororaphis* and *P. aureofaciens.*

Table 6. Trehalose and Adonitol Utilization of Pseudomonads.

Strains	Trehalose Utilization	Adonitol Utilization
P. fluorescens MB101	+	+
P. fluorescens Biovar I	+	+
P. fluorescens Biovar II	+	–
P. fluorescens Biovar IV	+	–
P. aeruginosa	–	–
P. putida Biovar A	–	–
P. putida Biovar B	–	–

The results of the biochemical tests listed above for *P. fluorescens* Biovars I, II & IV as well as for *P. aeruginosa* and *P. putida* Biovars A & B are from Bergey's Manual (1984).

Carbon Source Utilization Tests

Growth of strain MB101 on several carbon sources was used as a method of assigning this strain to a specific Biovar of *P. fluorescens.* When the growth characteristics of MB101 (Table 7) are compared with data compiled in Bergey's Manual (1984) the following inferences can be made:

- *P. putida, P. aeruginosa, P. chlororaphis,* and *P. aureofaciens* are clearly eliminated based on a large number of differences between their test results and those of MB101.
- Biovar IV can be eliminated based on erythritol and mesaconate utilization.
- Biovar II can be eliminated based on propylene glycol, ethanol and propanol utilization.

These results leave the possibility the MB101 may belong to *P. fluorescens* Biovars I, III and V. However, further phenotypic characterization of this strain indicates that *P. fluorescens* strain MB101 belongs to Biovar I since the denitrification results clearly eliminate Biovars II, III, and IV and the levan results clearly eliminate Biovar V.

Fatty Acid Methyl Ester Analysis

Fatty acids are long hydrocarbon chains ending in a carboxyl group that form an ester linkage when combined with glycerol, to form lipid molecules. These lipids are used to construct bacterial cell membranes. The diversity of structure of the fatty acids, their characteristic patterns within a taxon, and their stability (despite minor mutations) make them suitable for use in bacterial identification (Sasser, 1988). Whole-cell fatty acid methyl ester (FAME) profiles have been used in bacterial classification for over twenty years (Roy, 1988). This analysis of total bacterial cellular lipids by gas chromatography (GC) can be used to compare chromatograms of unknowns with those of known strains.

Fatty acid peak retention time, overall profiles and the ratios between fatty acids are used to group similar strains. Bacterial fatty acids are stable and reproducible when grown and processed under standardized conditions, regardless of geographic origin. Fatty acid-based groupings have thus far agreed with DNA homology data where available (Roy, 1988).

Table 8 provides the data for the FAME analysis and Figure 1 presents the FAME data in the form of a "nearest neighbor" analysis, which shows the relatedness of a single strain to another, or of a group of strains to another. The tree diagram shows that the recipient strain MB101 and its derivative MB214 are very closely related, and that they are both closest related to MB285. These three *P. fluorescens* strains are then most closely linked to MB237. These strains together form a cluster of very closely related *P. fluorescens* strains. Most of the *P. fluorescens* strains were shown to be closely related. One exception was strain MB295, which showed close relatedness to the *P. putida* group. Note that this strain has been described as atypical for the *P. fluorescens* species (Brosch *et al.*, 1996; see also below). Overall, these results confirm the results of the biochemical tests, which identify strain MB101 as a *P. fluorescens.*

Figure 1. Tree Diagram showing the results of the FAME analysis. The distance metric employed was EUCLIDIAN. The SINGLE LINKAGE method (nearest neighbor) was used.

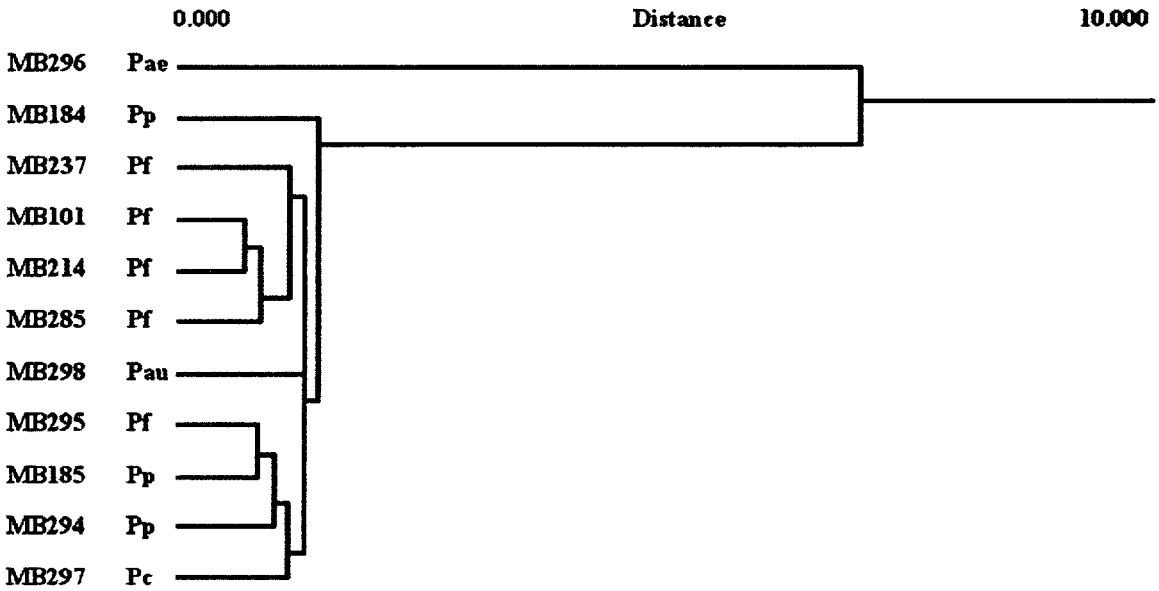

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Table 7. Carbon Utilization Tests for *Pseudomonas fluorescens*

COMPOUND	MB101	MB214	MB237 (Pf bv I)	MB285 (Pf)	MB295 (Pf bv I)	MB184 (Pp)	MB185 (Pp)	MB294 (Pp bv A)	MB296 (Pae)	MB297 (Pc)	MB298 (Pau)
D–Arabinose	–	–	–	–	–	–	–	–	–	–	–
D–Xylose	+	+	+	+	+	–	–	–	–	–	–
Saccharate	+	+	+	+	+	–	+	+	+	–	+
Mucate	+	+	+	+	+	–	+	–	+	+	+
Erythriol	+	+	–	+	+	–	–	–	–	–	–
Sorbitol	+	+	+	+	+	–	–	–	–	–	–
Propylene glycol	–	–	–	–	–	–	+	+	–	+	–
Malonate	+	+	+	+	+	–	+	+	+	+	+
Sebacate	–	–	–	–	–	–	–	–	+	–	–
Mesaconate	+	+	+	+	+	–	–	–	+	+	+
Hydroxymethylglutarate	+	+	+	+	+	–	–	–	–	–	–
Ethanol	–	–	–	–	–	–	+	+	–	+	–
Propanol	–	–	–	–	–	–	+	+	–	–	–
p–Hydroxybenzoate	+	+	+	+	+	+	+	+	+	+	+
Benzylformate	–	–	–	–	–	–	+	+	+	+	+
Glycine	–	–	–	–	–	+	+	+	–	–	–
L–Kynurenine	+	+	+	+	+	–	–	–	+	+	–
Kynurenate	+	+	–	+	–	–	–	–	–	+	–
Anthranilate	+	+	+	+	+	–	+	+	+	+	+
Benzylamine	–	–	–	–	–	+	+	+	–	+	–
Histamine	+	+	–	+	–	+	+	–	+	+	+
Tryptamine	–	–	–	–	–	–	+	–	–	–	–
Butylamine	–	–	–	–	–	+	+	+	+	–	+
α–Amylamine	–	–	+	–	–	+	+	+	+	–	–
Creatinine	–	–	–	–	–	–	+	+	–	–	–
Hippurate	–	–	–	–	–	–	+	+	–	–	–
Nicotinate	–	–	+	–	+	–	–	+	–	–	
Trigonelline	–	–	+	–	–	+	+	+	–	–	–

000084

Table 8. ***Pseudomonas*** **FAME Analysis. Numbers beneath each strain represent relative area (as %) under each peak.**

RT (min)	ID	MB101	MB184 (Pp)	MB185 (P p)	MB214	MB237 (Pf)	MB285 (Pf)	MB 294 (Pp)	MB295 (Pf)	MB296 (Pae)	MB297 (Pcl)	MB298 (Pau)
1.8		1.36	1.01	1.11	1.70	0.94	1.13	0.49	1.06	0.69	0.49	1.13
2.8				0.73								
3.9		0.98			1.12		1.02					
6.8	**n-C12:0**	4.01	7.10	3.48	4.47	4.87	5.17	3.62	5.58	5.21	2.34	2.77
10.0	**n-C14:0**	2.05	2.19	2.17	2.02	0.99	2.07	1.34	1.76	1.93	1.66	1.68
10.4		1.10			1.19	0.60	1.26	0.62				
11.7				0.61						0.61		
13.3	**n-C16:0**	31.07	27.01	30.48	31.83	32.59	29.47	31.99	29.08	22.56	34.18	32.48
13.6	**n-C16:1**			2.99		1.04		0.57	2.25	3.47	1.59	
13.7	**n-C16:1**	36.30	39.78	44.37	36.98	36.04	38.36	43.45	43.49	24.02	46.39	41.76
14.6								0.51		0.58	0.32	
15.2	**C17:0 isomer**	11.13	7.05	3.86	9.62	9.21	8.77	4.69	4.47	0.89	2.63	8.98
16.7										1.81		
17.4	**n-C18:0**	1.43	1.10		1.70	1.66	2.18	0.93	1.18		0.59	1.95
18.8	**n-C18:1**	7.80	12.04	8.93	8.47	12.06	7.74	10.11	9.34	34.11	9.04	7.70
21.5								0.51		2.62		
22.4	**C19:0 isomer**	2.79	2.73	1.29	0.89		2.80	1.19	1.81	1.52	0.77	1.57

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00648

4. GENOTYPIC CHARACTERIZATION

Identification of a Full-length 16S rRNA Gene in the Genome of MB214

A derivative of strain MB101 is MB214. The complete genome sequence of MB214 has been recently established. The *E. coli* 16S rRNA gene, *rrsE* (accession no.: ECRNA104) was employed to elucidate 16S rRNA genes from MB214 using the BLASTN function of GCG. These searches indicated that one copy of the 16S rRNA genes was fully sequenced. A binary alignment of the *E. coli* and *P. fluorescens* MB214 16S rRNA genes is provided in Figure 2.

Figure 2. Binary alignment of *E. coli* (top, capital letters) and *P. fluorescens* MB214 (bottom, small letters) 16S rRNA genes. The two sequences are 86% identical. The 3' ends of the two 16S rRNA genes that contain the anti-Shine-Dalgarno sequences are in bold red.

```
   1 AAATTGAAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTA 50
     || ||||||||||||||||||||||||||||||||||||||||||||||||
   1 gaactgaagagtttgatcatggctcagattgaacgctggcggcaggccta 50

  51 ACACATGCAAGTCGAACGGTAACAGGAAGAAGCTTGCTTCTTTGCTGACG 100
     ||||||||||||||| ||||   ||  |||||||||||||   ||   |
  51 acacatgcaagtcgagcggt...agagagaagcttgcttct...cttgag 94

 101 AGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGAT 150
     || |||||||||||||||||| || |||| ||||||| | | |||||||
  95 agcggcggacgggtgagtaatgcctaggaatctgcctggtagtgggggat 144

 151 AACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAG 200
     |||    ||||||||  |||||||||||||     |    |   ||||
 145 aacgttcggaaacggacgctaataccgcatacgtcctacgggagaaagca 194

 201 GGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTA 250
     |||||||||||||||   || ||| |||| ||| || | ||||||||||
 195 ggggaccttcgggccttgcgctatcagatgagcctaggtcggattagcta 244

 251 GTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGA 300
     || |||| ||||| |||||||| ||||||||||| || ||||||||||||
 245 gttggtgaggtaatggctcaccaaggcgacgatccgtaactggtctgaga 294

 301 GGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAG 350
     |||||| ||| ||||||||||||||||||||||||||||||||||||||
 295 ggatgatcagtcacactggaactgagacacggtccagactcctacgggag 344

 351 GCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGC 400
     |||||||||||||||||||| |||||||||| |||||||| |||||||||
 345 gcagcagtggggaatattggacaatgggcgaaagcctgatccagccatgc 394

 401 CGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGG 450
     ||||||| |||||||| |||||| |||||||| ||||| ||  ||||||
 395 cgcgtgtgtgaagaaggtcttcggattgtaaagcactttaagttgggagg 444

 451 AAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAG 500
     |||||    | ||||||| |      |||||||||||  ||||| |||
 445 aagggttgtagattaatactctgcaattttgacgttaccgacagaataag 494

 501 CACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCG 550
     |||||||||||| |||||||||||||||||||||| |||||||||||||
 495 caccggctaactctgtgccagcagccgcggtaatacagagggtgcaagcg 544

 551 TTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCA 600
     |||||||||||||||||||||||||||||| || ||| ||||||||||||
 545 ttaatcggaattactgggcgtaaagcgcgcgtaggtggtttgttaagttg 594

 601 GATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAG 650
     |||||||||||||||||||||||||||||||||||   | |||| |
 595 gatgtgaaatccccgggctcaacctgggaactgcattcaaaactgactga 644

 651 CTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCG 700
     || |||| | |||||||| ||| ||||| |  ||||||||||||||||||
 645 ctagagtatggtagagggtggtggaatttcctgtgtagcggtgaaatgcg 694

 701 TAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGAC 750
     |||| ||  | ||||| ||| |||||||||| || ||||||| || ||
 695 tagatataggaaggaacaccagtggcgaaggcgaccacctggactaatac 744

 751 TGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGG 800
     |||| || |||||||||||||||||||||||||||||||||||||||||
 745 tgacactgaggtgcgaaagcgtggggagcaaacaggattagataccctgg 794

 801 TAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGT 850
     |||||||||||||||||||||||| ||| |  | | |   |||||||  |
 795 tagtccacgccgtaaacgatgtcaactagccgttggaagccttgagcttt 844

 851 GGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCA 900
       |  || ||||||||| |||||| |||||||||||||||||||||||||
 845 tagtggcgcagctaacgcattaagttgaccgcctggggagtacggccgca 894

 901 AGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT 950
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 895 aggttaaaactcaaatgaattgacgggggcccgcacaagcggtggagcat 944

 951 GTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCA 1000
     ||||||||||||||| |||||||||||||||||||||| || ||||||||||
 945 gtggtttaattcgaagcaacgcgaagaaccttaccaggccttgacatcca 994

1001 CGGAAGTTTTCAGAGATGAGAATGTGCCTTCGGGAACCGTGAGACAGGTG 1050
       ||| |||  ||||||      |||||||||||||||  ||||||||||
 995 atgaactttctagagatagattggtgccttcgggaacattgagacaggtg 1044

1051 CTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGC 1100
     |||||||||||||||||||||||||||| |||| ||||||||||||||||
1045 ctgcatggctgtcgtcagctcgtgtcgtgagatgttgggttaagtcccgt 1094

1101 AACGAGCGCAACCCTTATCCTTTGTTGCCAGCG.GTCCGGCCGGGAACTC 1149
     |||||||||||||||| ||||| ||| |||||| ||   |  ||| ||||
1095 aacgagcgcaacccttgtccttagttaccagcgcgtaatggtgggcactc 1144

1150 AAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGT 1199
      ||||||||||||| |||| |||| |||||||||||||||||||||||||
1145 taaggagactgccggtgacaaaccggaggaaggtggggatgacgtcaagt 1194

1200 CATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCATAC 1249
     |||||||||||||||||| || |||||||||||||||||||||||    |||
1195 catcatggcccttacggcctgggctacacacgtgctacaatggtcggtac 1244

1250 AAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTA 1299
     | || |  || |   ||||||    |||  | | ||||||     |||||
1245 agagggttgccaaaccgcgaggtggagctaatcccataaaaccgatcgta 1294

1300 GTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTA 1349
     |||||||| | |||||||||||||||| | |||||||||||||||||||
1295 gtccggatcgcagtctgcaactcgactgcgtgaagtcggaatcgctagta 1344

1350 ATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACAC 1399
     |||| | ||||||||| | |||||||||||||||||||||||||||||||
1345 atcgcgaatcagaatgtcgcggtgaatacgttcccgggccttgtacacac 1394

1400 CGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACC 1449
     |||||||||||||||||||||||||||||||||||  |||||||| |||  |||||
1395 cgcccgtcacaccatgggagtgggttgcaccagaagtagctagtctaacc 1444

1450 TTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGTA 1499
     ||||||||| || |||||||  |||||||||||||||||||||||||||||
1445 ttcgggaggacggttaccacggtgtgattcatgactggggtgaagtcgta 1494

1500 ACAAGGTAACCGTAGGGGAACCTGCGGTTGGATCACCTCCTTA 1542
     |||||||| |||||||||||||||||||| |||||||||||||||
1495 acaaggtagccgtaggggaacctgcggctggatcacctcctta 1537
```

000086

DNA Sequencing of the 16S rRNA Genes of MB101 and DC88

Strain MB101 is the parental strain of MB214 and the recipient strain used in construction of the production strain DC88. To confirm direct ancestral lineage of DC88 from the parental strain, MB101, the sequences of the 16S rRNA genes of MB101 and DC88 were compared (see Material and Methods section). The sequence analysis showed that the 16S rRNA DNA sequences of MB101 and DC88 were identical to that of MB214 (data not shown). These data confirm that no strain mix-up has occurred and the direct ancestral lineage of DC88 from MB101.

Phylogenetic Analysis of MB101 and DC88 based on 16S rRNA Genes

Moore *et al.* (1996) used the 16S rRNA gene sequences to identify species of the genus *Pseudomonas*. They sequenced 16S rRNA genes from 24 different *Pseudomonas* species and found that only 148 positions of the 1492 bp sequenced in each strain were variable. Sixty-five of these 148 variable nucleotides were found to be located in three small regions, which are referred to as hyper-variable regions 1, 2, and 3. Analysis of these hyper-variable regions enabled differentiation into different *Pseudomonas* species and to a certain extent into specific Biovars.

The three hyper-variable regions from MB101 and DC88 were compared to the hyper-variable regions of several *Pseudomonas* species. Figure 3 shows the multiple sequences alignment of **hyper-variable regions 1** with other strains in the *P. fluorescens* lineage, which was found to be closest related. The sequences from other *Pseudomonas* lineages are not shown due to their sequence dissimilarities.

Figure 3. Comparison of the hyper-variable region 1 of strains from the *P. fluorescens* lineage.

Strain	Sequence	*P. fluorescens* Lineage
MB101	gcggtagagagaagcttgcttctcttgagagc	TBD
DC88	gcggtagagagaagcttgcttctcttgagagc	TBD
MB295	gcggtagagagaagcttgcttctcttgagagc	*P. fluorescens*
LMG2210	gcggtagagagaagcttgcttctcttgagagc	*P. marginalis*
LMG2342	gcggtagagagaagcttgcttctcttgagagc	*P. tolaasii*
DSM6698	gcggtagagagaagcttgcttctcttgagagc	*P. aureofaciens*
LMG5004	gcggtagagag**gt**gcttgc**ac**ctcttgagagc	*P. chlororaphis*
LMG2352	gcggtagagagaagcttgcttctcttgagagc	*P. viridiflava*

Deviating residues are in bold red. TBD: to be determined.

The DNA sequences of hyper-variable region 1 were identical in five of the six species of the *Pseudomonas fluorescens* lineage analyzed by Moore *et al.* (1996). Therefore, the DNA sequence in the hyper-variable region 1 cannot distinguish most of the species in that lineage. Based upon the DNA sequences of hyper-variable region 1, strains MB101 and DC88 could not be differentiated from the following *Pseudomonas* species: *fluorescens*, *marginalis*, *tolaasii*, *aureofaciens*, or *viridiflava*.

Comparison of **hyper-variable regions 3** of the pseudomonads showed that the sequences of strains MB101 and DC88 were identical to the *P. fluorescens* species only (Figure 4). Therefore, hyper-variable region 3 was able to distinguish the species of the *P. fluorescens* lineage from each other, as well as from all other *Pseudomonas* species. This finding that the DNA sequences of hyper-variable

region 3 were identical further support the results of the phenotypic characterization that MB101 and DC88 are indeed *P. fluorescens.*

Figure 4. Comparison of the hyper-variable regions 3 of strains from the *P. fluorescens* lineage.

Strain	Sequence	*P. fluorescens* Lineage
MB101	atccaatgaactttctagagatagattggtgccttcgggaacattgaga	TBD
DC88	atccaatgaactttctagagatagattggtgccttcgggaacattgaga	TBD
MB295	atccaatgaactttctagagatagattggtgccttcgggaacattgaga	*P. fluorescens*
LMG2210	atccaatgaactttc**c**agagat**g**gattggtgccttcgggaacattgaga	*P. marginalis*
LMG2342	atccaatgaactttc**c**agagat**g**gattggtgccttcgggaacattgaga	*P. tolaasii*
DSM6698	atccaatgaactttc**c**agagat**g**gattggtgccttcgggaacattgaga	*P. aureofaciens*
LMG5004	atccaatgaactttc**c**agagat**g**gattggtgccttcgggaacattgaga	*P. chlororaphis*
LMG2352	atccaatgaa**tcc**t**t**tagagataga**gga**gtgccttcgggag**g**cattgaga	*P. viridiflava*

Deviating residues are in bold red. TBD: to be determined.

The DNA sequences within **hyper-variable region 2** are able to differentiate to a limited extent the various Biovars within the *P. fluorescens* species. In order to be more accurate in their analyses, Moore *et al.* (1996) sequenced and compared the hyper-variable regions of additional strains of species belonging to the *P. fluorescens* lineage including four of the five Biovars of *P. fluorescens*. Figure 5 shows the multiple sequences alignment for hyper-variable regions 2 of the analyzed *P. fluorescens* Biovars, which were found to be closest related. The sequences from the other species are not shown.

Figure 5. Comparison of the hyper-variable regions 2 of strains from the species *P. fluorescens*.

Strain	Sequence	*P. fluorescens* Biovar
MB101	gggttgtagattaatactctgcaattt	TBD
DC88	gggttgtagattaatactctgcaattt	TBD
LMG5825	gggttgtagattaatactctgcaattt	Biovar I
LMG5830	gggttgtagattaatactctgcaattt	Biovar I
MB295	ggg**cat**ta**acc**taatac**gt**t**agtg**ttt	Biovar I
LMG5916	ggg**ca**gt**tacc**taatac**gtgattg**ttt	Biovar I
LMG1244	gggttgtagattaatactctgcaattt	Biovar III
LMG5822	gggttgtagattaatactctgcaattt	Biovar III
LMG5831	gggttgtagattaatactctgcaattt	Biovar III
LMG5938	ggg**ca**gta**a**attaatact**t**tgc**tg**ttt	Biovar III
LMG5939	ggg**ca**gt**tacc**taatac**gtatc**t**g**ttt	Biovar IV
LMG5167	ggg**ca**gt**tacc**taatac**gtgattg**ttt	Biovar V
LMG5833	ggg**ca**gt**tacc**taatac**gtgattg**ttt	Biovar V
LMG5940	ggg**ca**gt**tacc**taatac**gtgattg**ttt	Biovar V

Deviating residues are in bold red. TBD: to be determined.

Analysis of the multiple sequence alignment indicates that MB101 and DC88 comprise the same hyper-variable regions 2 as some of the *P. fluorescens* Biovars I and III. Interestingly, the DNA sequence within Biovar I is not conserved deviating in as many as 13 positions. MB295 is the recognized type strain for the *P. fluorescens* species. However, not a single other strain of *P. fluorescens* in the study by Moore *et al.* (1996) was found to possess the same sequence as MB295. This strain has been noted as atypical for the *P. fluorescens* species before (Brosch *et al.* 1996; see also FAME relatedness analysis in Figure 1 above). Taken together, the morphologic, phenotypic and

genotypic characterizations, it is concluded that MB101 and DC88 both belong to *Pseudomonas fluorescens* Biovar I.

DISCUSSION

Numerous independent biochemical and genotypic analyses clearly demonstrate that strain MB101 is appropriately assigned to *Pseudomonas fluorescens* Biovar I.

Strains MB101 and DC88 belong to the species *Pseudomonas fluorescens*
Strain MB101 was independently identified as a *P. fluorescens* by API rapid NFT tests that were conducted in the laboratory of Professor I.J. Misaghi, as well identified by company personnel (Table 3). In both cases, good identification numbers were obtained with a confidence of 99.8%. A battery of other biochemical tests was performed, which included production of levan and phenizine, presence or absence of lecithinase, ability to denitrify and hydrolyze gelatin, utilization of trehalose and numerous other carbon sources, and analysis of fatty acid methyl esters. The results of all these tests corroborated the finding by API rapid NFT tests that MB101 is *P. fluorescens* (Tables 4 to 7). The following biochemical characteristics demonstrate that MB101 does **not** belong to the *Pseudomonas* species *aeruginosa*, *putida*, *chlororaphsis* and *aureofaciencs*:

- Test results of MB101, which disagree with those of *P. aeruginosa*, include levan production, phenizine production, lecithinase, denitrification, gelatin hydrolysis and adonitol utilization, thus eliminating *P. aeruginosa* as a viable identity candidate.
- Test results of MB101, which disagree with those of *P. putida*, include levan production, lecithinase and adonitol utilization, thus eliminating this species as a likely identity candidate.
- Test results of MB101, which disagree with those of *P. chlororaphis*, include phenizine production, denitrification, gelatin hydrolysis, and adonitol utilization thus eliminating it as a likely identity candidate.
- Test results of MB101, which disagree with those of *P. aureofaciens*, include phenizine production, gelatin hydrolysis and adonitol utilization.

In addition to phenotypic characterizations, genotypic analyses using the 16S rRNA genes from MB101 and DC88 were carried out. Hyper-variable regions within the 16S rRNA gene sequences were used previously to differentiate between different *Pseudomonas* species (Moore *et al.*, 1996). Comparison of the hyper-variable regions of MB101 and DC88 to the hyper-variable regions of other *Pseudomonas* species showed that both MB101 and DC88 were most closely related to *P. fluorescens*. Thus, both phenotypic and genotypic analyses support the notion that MB101 and DC88 are *P. fluorescens* strains.

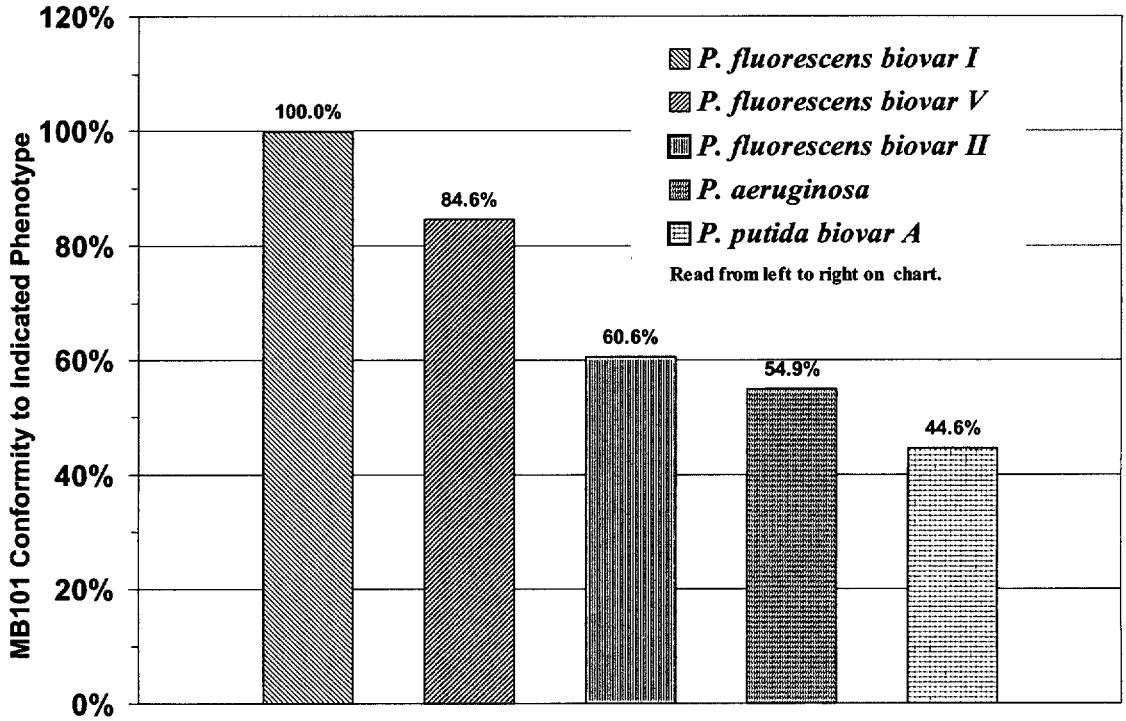
Strains MB101 and DC88 belongs to Biovar I
Analysis of the 16S rRNA from MB101 and DC88 showed that the two strains comprise the same hyper-variable regions 2 as some of the *P. fluorescens* Biovars 1 and 3, but deviate substantially from the other *P. fluorescens* Biovars analyzed. These findings suggest that MB101 and DC88 may belong to either Biovar I or III.

Growth on several carbon sources, however, allowed determination of the specific *P. fluorescens* Biovar. The results of these growth tests demonstrated that *P. fluorescens* Biovars II and IV could be

eliminated due to their growth differences in minimal medium supplemented with various carbon sources. The *P. fluorescens* Biovars III and V were eliminated based on their denitrification and levan production results. Based upon these results strain MB101 can be classified as *Pseudomonas fluorescens* Biovar I.

A graphic representation of the results of the overall biochemical analysis is presented in Figure 6. Phenotypes of control strains for *P. fluorescens* Biovar I, *P. putida, P. aeruginosa, P. fluorescens* Biovar II and *P. fluorescens* Biovar V were obtained from Bergey's Manual (1984). Only those reactions that were rated an unambiguous + (positive) or - (negative) were included. Growth reactions of strain MB101 were compared to the test results shown from Bergey's Manual and a percentage of conformity was calculated. As can be seen from Figure 6, the *P. fluorescens* Biovar I shows the closest conformity with our test results on MB101. Next closest was *P. fluorescens* Biovar V and next was *P. fluorescens* Biovar II. The least amount of conformity to the test strain results was shown by the *P. putida* Biovar A and the *P. aeruginosa* information. This graphically illustrates the results obtained in identification of the MB101 recipient strain as *Pseudomonas fluorescens* Biovar I.

Figure 6. Phenotypic conformity of Strain MB101 to reference strains.



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6. Discovery Article

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7. Plasmid Maps

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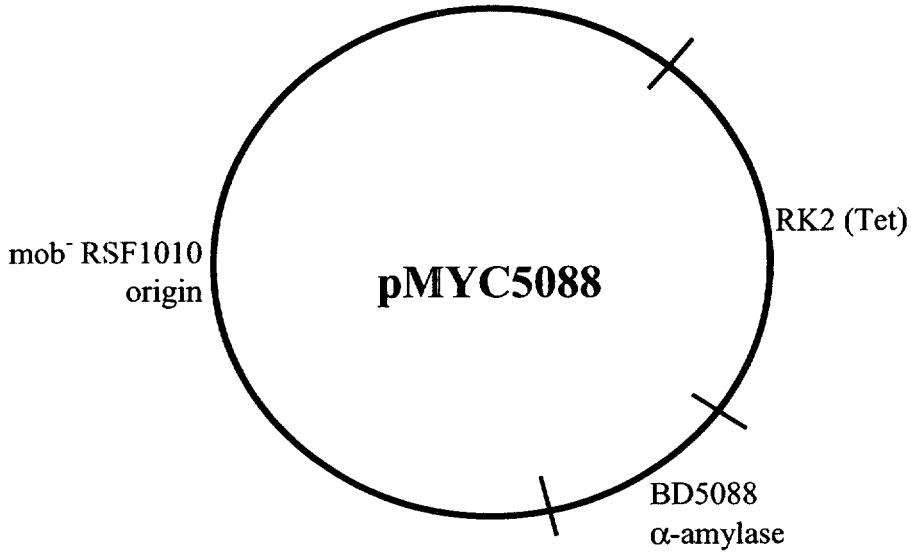
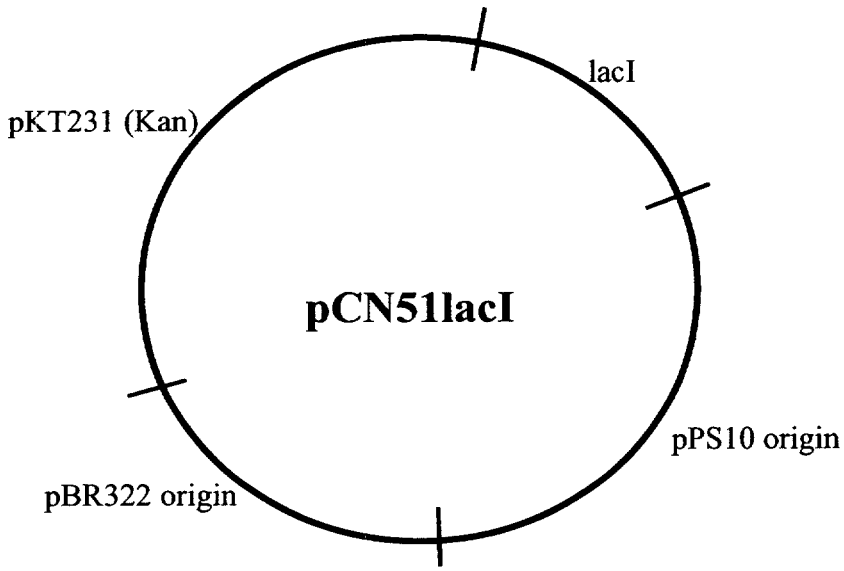
Appendix 7

Plasmid Maps: Plasmids pMYC5088 and pCN51lacI





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Appendix 8

BD5088 Amino Acid Sequence Analysis

INTRODUCTION

Appendix 8 presents two sections, the first (8.a) compares the amino acid sequence of BD5088 with those of other native α-amylases from a diverse variety of sources, and the second (8.b) focuses more specifically on amino acid sequence comparisons between BD5088 and its archaeal parental genes with other native archaeal α-amylases.

8.a Comparison of BD5088 to Native α-Amylases of Various Phylogenic Domains

Figure 1 is an alignment of α-amylase amino acid sequences chosen from very different phylogenetic organisms of various phylogenic domains; bacteria, mammals, plant, bacteria and fungi are all compared to BD5088. All of these α-amylases belong to glycosyl hydrolase family 13 as described by Henrisaatt and Davies (1997). The sequences were aligned using the program ClustalW using standard parameters and implemented through Vector NTI (Informax).

Figure 1.

```
                          1                                                  50
BD5088          (1) ------------------------------MAKYSELEKGG----------
A.oryzae        (1) -------------MMVAWWSLFLYGLQVAAPALAATPADWRSQSIYFLLT
B.licheniformis     (1) ------MKQQKRLYARLLTLLFALIFLLPHSAAAAANLNG----------
B.stearothermophilus (1) MLTFHRIIRKGWMFLLAFLLTVSLFCPTGQPAKAAAPFNG----------
human           (1) --------------MKLFWLLFTIGFCWAQYSSNTQQGRT----------
potato          (1) --------------------MALDESQQSDPLVVIRNGKE----------
RAT             (1) ----MREYCQHHSRMKFFLLLSLIGLCWAQYDPHTFYGRS----------
Consensus       (1)                M  F LL  I    A  A A   G

                          51                                                100
BD5088         (12) ---------VIMQAFYWDVPSGGIWWDTIRQKIPEWYDAGISAIWIPPAS
A.oryzae       (38) DRFARTDGSTTATCNTADRKYCGGTWQGIIDKLDYIQGMGFTAIWITPVT
B.licheniformis     (35) ---------TLMQYFEWYMPNDGQHWKRLQNDSAYLAEHGITAVWIPPAY
B.stearothermophilus (41) ---------TMMQYFEWYLPDDGTLWTKVANEANNLSSLGITAIWLPPAY
human          (27) ---------SIVHLFEWRWVDIALECER------YLAPKGFGGVQVSPPN
potato         (21) ---------IILQAFDWES-HKHDWWLNLDTKVPDIAKSGFTTAWLPPVC
RAT            (37) ---------SIVHLFEWRWVDIAKECER------YLAPNGFGGVQVSPPN
Consensus      (51)           TIMQ FEW     G  W RI     YLA  GFTAVWIPP

                          101                                               150
BD5088         (53) KGMGGAYSMGYDPYDFFDLGEYDQKGTVETRFGSKQELVNMINTAHAYGM
A.oryzae       (88) AQLPQTTAYGDAYHGYWQQDIYS----LNENYGTADDLKALSSALHERGM
B.licheniformis     (76) KGTS-QADVGYGAYDLYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDI
B.stearothermophilus (82) KGTS-RSDVGYGVYDLYDLGEFNQKGTVRTKYGTKAQYLQAIQAAHAAGM
human          (62) ENVA-IHNPFRPWWERYQPVSYK----LCTRSGNEDEFRNMVTRCNNVGV
potato         (61) QSLAPEGYLPQNLYS------------LNSKYGSEDLLKALLNKMKQYKV
RAT            (72) ENIV-VNSPFRPWWERYQPISYK----ICSRSGNEDEFRDMVNRCNNVGV
Consensus     (101)   LA    VG   YD Y    Y     L TKYGT DELK MIN  H  GM
```

000103

Conserved Region 1

```
                                  151                                              200
                 BD5088   (103)   KVIADIVINHRAGGDLEWNPFVNDYTWTDFSKVASG--------------
               A.oryzae   (134)   YLMVDVVANHMGYDGAGSSVDYSVFKPFSSQD------------------
        B.licheniformis   (125)   NVYGDVVINHKGGADATEDVTAVEVDPADRNRVISGEHLIKAWTHFHFPG
   B.stearothermophilus   (131)   QVYADVVFDHKGGADGTEWVDAVEVNPSDRNQEISGTYQIQAWTKFDFPG
                  human   (107)   RIYVDAVINHMCGNAVSAGTSSTCGSYFNPG------------SR-DFP-
                 potato    (99)   RAMADIVINHRVGTTQGHGGMYNRYDGIPMS-------------------
                    rat   (117)   RIYVDAVINHMCGVGAEAGQSSTCGSYFNPN------------NR-DFP-
              Consensus   (151)   RVYADVVINHKGG   A      A          N             FP

                                  201                                              250
                 BD5088   (139)   ------------------------KYTANYLDFHPNELHAGDSGTFGG--
               A.oryzae   (166)   -------------------------YFHPFCLIQNYEDQTQVEDCWLG-D
        B.licheniformis   (175)   RGSTYSDFKWHWYHFDGTDWDESRKLNRIYKFQ--GKAWDWEVSNENGNY
   B.stearothermophilus   (181)   RGNTYSSFKWRWYHFDGVDWDESRKLSRIYKFRGIGKAWDWEVDTENGNY
                  human   (143)   -A---------VP-YSGWDFNDGKCKTGSGDIENYNDATQVRDCRLSG--
                 potato   (130)   ------------------------WDEHAITSCTGGRGNKSTGDNFNG--
                    rat   (153)   -G---------VP-YSGFDFNDGKCKTGSGGIENYNDAAQVRDCRLSG--
              Consensus   (201)    G              F G DF D K  T  Y       DA      G
```

Conserved Region 2

```
                                  251                                              300
                 BD5088   (163)   ----YPDICHDKSWDQYWLWASQESYAAYLRSIGIDAWRFDYVKGYAPWV
               A.oryzae   (190)   NTVSLPDLDTTKDVVKNEWYDWVG---SLVSNYSIDGLRIDTVKHVQKDF
        B.licheniformis   (223)   DYLMYADIDYDHPDVAAEIKRWG---TWYANELQLDGFRLDAVKHIKFSF
   B.stearothermophilus   (231)   DYLMYADLDMDHPEVVTELKNWG---KWYVNTTNIDGFRLDAVKHIKFSF
                  human   (180)   ----LLDLALGKDYVRSKIAEY----MNHLIDIGVAGFRIDASKHMWPGD
                 potato   (154)   ----VPNIDHTQSFVRKDLIDWMR---WLRSSVGFQDFRFDFAKGYASKY
                    rat   (190)   ----LLDLALEKDYVRTKVADY----MNHLIDIGVAGFRLDASKHMWPGD
              Consensus   (251)       L DLD DK YVR EL DW        YL  IGIDGFRLDAVKHI   F
```

Conserved Region 3

```
                                  301                                              350
                 BD5088   (209)   VKDWLNWWG------------GWAVGEYWDTNVDA--------------V
               A.oryzae   (237)   WPGYNKAAG------------VYCIGEVLDGDPAY---------------
        B.licheniformis   (270)   LRDWVNHVR------EKTGKEMFTVAEYWQNDLGA--------------L
   B.stearothermophilus   (278)   FPDWLSYVR------SQTGKPLFTVGEYWSYDINK--------------L
                  human   (222)   IKAILDKLHNLNSNWFPEGSKPFIYQEVIDLGGEP--------------I
                 potato   (197)   VKEYIEGAEP-----------IFAVGEYWDTCNYKGSNLDYNQDSHRQRI
                    rat   (232)   IKAVLDKLHNLNTKWFSEGSKPFIYQEVIDLGGEA--------------V
              Consensus   (301)   IKDWL  L            G  IF VGEYWD                  I

                                  351                                              400
                 BD5088   (233)   LNWAYSSGAKVFDFALYYKMDEAFDNK-NIPALVSALQNGQTVVSRDPFK
               A.oryzae   (260)   --TCPYQNVMDGVLNYPIYYPLLNAFKSTSGSMDDLYNMINTVKSDCPDS
        B.licheniformis   (300)   ENYLNKTNFNHSVFDVPLHYQFHAAST-QGGGYDMRKLLNGTVVSKHPLK
   B.stearothermophilus   (308)   HNYITKTDGTMSLFDAPLHNKFYTASK-SGGAFDMRTLMTNTLMKDQPTL
                  human   (258)   KSSDYFGNGRVTEFKYGAKLGTVIRKW-NGEKMSYLKNWGEGWGFMPSDR
                 potato   (236)   INWIDGAGQLSTAFDFTTKAVLQEAVKGEFWRLRDSKGKPPGVLGLWPSR
                    rat   (268)   SSNEYFGNGRVTEFKYGAKLGKVLRKW-DGEKMAYLKNWGEGWGFMPSDR
              Consensus   (351)    NW    NG VT F Y  K      A K  G  M   KN    TVV   P R
```

000104

Conserved Region 4

```
                                401                                                450
BD5088                  (282)   AVT--FVANHDTDI--------------IWNKYPAYAFILTYE-GQPTIF
A.oryzae                (308)   TILGTFVENHDNPR-------FASYTNDIALAKNVAAFIILND-GIPIIY
B.licheniformis         (349)   SVT--FVDNHDTQP-----GQSLESTVQTWFKPLAYAFILTRESGYPQVF
B.stearothermophilus    (357)   AVT--FVDNHDTEP-----GQALQSWVDPWFKPLAYAFILTRQEGYPCVF
human                   (307)   AIV--FVDNHDNQRGHGAGGASILTFWDARLYKMAVGFMLAHPYGFTRVM
potato                  (286)   AVT--FIDNHDTGS------TQAHWPFPSRHVMEGYAYILTHP-GIPSVF
rat                     (317)   AIV--FVDNHDNQRGHGAGGSSILTFWDARLYKMAVGFMLAHPYGFTRVM
Consensus               (401)   AVT  FVDNHDT       G SI S  D     LAYAFILTH  GFP VF

                                451                                                500
BD5088                  (315)   YRDYEEWLN---------KD--------KLKNLIWIHENLAGG-------
A.oryzae                (350)   AGQEQHYAGGNDPANREATWLSGYPTDSELYKLIASANAIRNYAIS----
B.licheniformis         (392)   YGDMYGTKGDS-----QREIPA---LKHKIEPILKARKQYAYG-------
B.stearothermophilus    (400)   YGDYYGIP--------QYNIPS---LKSKIDPLIARRDYAYG-------
human                   (355)   SSYRWPRYFENGKDVNDWVGPP---NDNGVTKEVTINPDTTCGNDWVCEH
potato                  (327)   FDHFYEWDN------------S---MHDQIVKLIAIRRNQGIHS------
rat                     (365)   SSFHWPRYFENGKDVNDWVGPP---NNNGATKEVTINSDSTCGNDWVCEH
Consensus               (451)   Y   Y                PS       I KLI I    A G

                                501                                                550
BD5088                  (341)   --------STDIVYYD-NDELIFVRNGYGDKP------GLITYINLGSSK
A.oryzae                (396)   ---------KDTGFVTYKNWPIYKDDTTIAMRKGTDGSQIVTILSNKGAS
B.licheniformis         (427)   ---------AQHDYFDHHDIVGWTREGDSSVANS----GIAALITDGPGG
B.stearothermophilus    (432)   ---------TQHDYLDHSDIIGWTREGGTEKPGS----GIAALITDGPGG
human                   (402)   RWRQIRNMVNFRNVVDGQPFTNWYDNGSNQVAFGRGNRGFIVFNNDDWTF
potato                  (356)   ----------------------------------------------R--SS
rat                     (412)   RWRQIRNMVAFRNVVNGQPFANWWDNGSNQVAFGRGNKGFIVFNNDDWDL
Consensus               (501)                YVD      W   G   V       GLI  I D

                                551                                                600
BD5088                  (376)   AGRWVYVPKFAGACIHEYIGNLGG----WVDKYVYSSGWVYLEAPAYDPA
A.oryzae                (437)   GDSYTLSLSGAGYTAGQQLTEVIG--CTTVTVGSDGNVPVPMAGGLPRVL
B.licheniformis         (464)   AKRMYVGRQNAGETWHDITGNRSE------PVVINSEGWGEFHVNGGSVS
B.stearothermophilus    (469)   SKWMYVGKQHAGKVFYDLTGNRSD------TVTINSDGWGEFKVNGGSVS
human                   (452)   SLTLQTG-LPAGTYCDVISGDKINGNCTGIKIYVSDDGKAHFSISNSAED
potato                  (359)   IRILEAQPNLYAATIDEKVSVKIG-------DGS-----WSPAGKEWTLA
rat                     (462)   STTLQTG-LPAGTYCDVISGDKVDGNCTGIKVYVGSDGNAYFSISNSAED
Consensus               (551)   S  L  G   AG    DITG KI      I V V SDG A FAI   AVA

                                601                          637
BD5088                  (422)   NGQYGYSVWSYCGVG-----------------------
A.oryzae                (485)   YPTEKLAGSKICSSS-----------------------
B.licheniformis         (508)   IYVQR---------------------------------
B.stearothermophilus    (513)   VWVPRKTTVSTIARPITTRPWTGEFVRWTEPRLVAWP
human                   (501)   PFIAIHAESKL--------------------------
potato                  (397)   TSGHRYAVWQK--------------------------
rat                     (511)   PFIAIHVESKI--------------------------
Consensus               (601)    FI RHA
```

000105

Table 1 provides the % amino acid distance between the aligned sequences. The algorithm (Distances) examines each pair of aligned sequences symbol-by-symbol and counts the number of exact matches, partial matches and gap symbols. The Distances program is within the Genetics Computer Group (GCG) sequence analysis software package (version 9.0).

Table 1. Amino Acid Distance Between Aligned Sequences.

Amylase Source	BD5088	*A. oryzae*	*B. licheni-formis*	*B. stearo-thermophilus*	Human	Potato	Rat
BD5088	**100**	16	30	30	16	27	17
A. oryzae		**100**	18	19	16	21	16
B. licheniformis			**100**	63	17	19	18
B. stearothermophilus				**100**	16	19	17
Human					**100**	16	86
Potato						**100**	16
Rat							**100**

Table 2 examines the four highly conserved regions that are common to all α-amylases of family 13. It demonstrates that BD5088 is similar at the amino acid level to known α-amylases including those that are used commercially currently (*B. licheniformis*). BD5088 is 72% identical to the consensus sequence (18/25 residues) for the 4 conserved regions, and 92% similar (23/25 residues). There is also precedent in the literature that suggests that the archaeal α-amylases such as BD5088 are more closely related to plant α-amylases as compared to mammalian enzymes (Janecek *et al.*, 1999).

Table 2. Comparison of Alpha-Amylase Highly Conserved Regions.

Amylase Source	Region 1	Region 2	Region 3	Region 4
BD5088	DIVINH	AWRFDYVKG	EYWD	FVANHD
Potato	DIVINH	DFRFDFAKG	EYWD	FIDNHD
A. oryzae	DVVANH	GLRIDTVKH	EVLD	FVENHD
B. stearothermophilus	DVVFDH	GFRLDAVKH	EYWS	FVDNHD
B. licheniformis	DVVINH	GFRLDAVKH	EYWQ	FVDNHD
Rat	DAVINH	GFRLDASKH	EVID	FVDNHD
Human	DAVINH	GFRIDASKH	EVID	FVDNHD
Consensus	DVVINH	GFRLDAVKH	EYWD	FVDNHD

BD5088	DIVINH	AWRFDYVKG	EYWD	FVANHD
Consensus	DVVINH	GFRLDAVKH	EYWD	FVDNHD
Similarity	*:****	::*.* **	****	** ***

* = identical, : = change to functionally identical amino acid, . = conservative amino acid change.

000106

8.b Comparison of BD5088 to Native Archaeal α-Amylases

Over the past decade there have been several reports that have described the isolation and characterization of secreted α-amylases from thermophilic archaea. These enzymes have originated from two Genus of bacteria– *Thermococcus* and *Pyrococcus*. These organisms have the following taxonomic lineage as described in Bergey's Manual of Systematic Bacteriology (2001):

Domain: Archaea
Phylum: Euryarchaeota
Class V: Thermococci
Order I: Thermococcales
Genus I: *Thermococcus*
Genus II: *Pyrococcus*

Typical characteristics of Genus I (*Thermococcus*) organisms are that they are strictly anaerobic and extremely thermophilic with optimal temperatures for growth between 75 and 88°C. NaCl is required for growth with optimal concentrations between 2 and 4%. They have been found to inhabit coastal and submarine solfataras, including deep-sea hydrothermal vents. They have also been isolated from New Zealand terrestrial thermal springs (Bergey's Manual, 2001).

Typical characteristics of Genus II (*Pyrococcus*) organisms are that they are strictly anaerobic and temperature for growth is between 67-103°C with an optimum of 100°C. Species of *Pyrococcus* have been found in deep-sea hydrothermal vents and from sediments in vents of marine solfataras (Bergey's Manual, 2001).

It is difficult to classify archaea as either gram positive or gram negative as they do not typically have the cell wall mureins required for staining. Because of the lack of staining, one might classify them as gram negatives; however, it is not a true indication, as the stain was originally intended for eubacteria and not archaea.

To date there have been six reports of secreted hyperthermophilic α-amylases, three from *Pyrococcus* species:

1) *Pyrococcus woesei* (Koch *et al.*, 1991)
2) *Pyrococcus furiosus* (Dong *et al.*, 1997)
3) *Pyrococcus sp.* KOD1 (Tachibana *et al.*, 1996)

and three from *Thermococcus* species:

1) *Thermococcus sp.* Rt3 (Jones *et al.*, 1999)
2) *Thermococcus hydrothermalis* (Lévêque *et al.*, 2000a)
3) *Thermococcus profundus* (Chung *et al.*, 1995; Lee *et al.*, 1996).

000107

The characteristics of these α-amylases have been summarized in a recent review by Lévêque *et al.*, (2000b). In brief, these enzymes for the most part, do not differ from their mesophilic counterparts in molecular weight or amino acid composition. In general, the *Thermococcus* α-amylases tend to be less thermophilic than their *Pyrococcus* counterparts as reflected in the differences in optimal growth

temperatures of these organisms. In this same review, alignments of the thermophilic archaeal α-amylase amino acid sequences show that they have >75% similarity.

To date, there have been no reports of an opportunistic archaeal infection (Reeve, 1999). Additionally, to date, all of the species from which this type of α-amylase has arisen only grow above 67°C.

The three α-amylase genes used to develop BD5088 came from three different sources. Two of the genes, BD5031 and BD5064, were isolated from libraries constructed from pure cultures of Thermococcus organisms. BD5063 originated from a library constructed from a primary enrichment containing an undetermined number of high temperature organisms. A detailed description of the discovery and optimization of BD5088 has been described by Richardson, *et. al.* (2001). This paper can be found as well in Appendix 6.

BD5031
This gene was isolated from a proprietary species of *Thermococcus* AEPII1a that is not available in any publicly available culture collection. The species was isolated from a sample taken from a shallow marine hydrothermal system, temperature of 95°C and pH 7.0, by Professor Karl O. Stetter (Institute for Microbiology, University of Regensburg; author of pertinent sections on Genus II *Pyrococcus* in Bergey's Manual (2001).

BD5064
This gene was isolated from a proprietary species of *Thermococcus* GU5L5 that is not available in any publicly available culture collection. The species was isolated from a sample taken at a shallow marine hydrothermal system, temperature of 85°C and pH 6.0, by Professor Karl O. Stetter.

BD5063
This gene was isolated from a library that had been constructed from DNA isolated from a primary enrichment culture, from a sample taken from the Deep Sea Pacific Ocean, temperature of 90°C and pH 6.5, by Dr. Martin Keller (Diversa Corporation). The original sample contained a variety of thermophilic organisms, including organisms from the Order Thermococcales, as determined by 16S rRNA analysis of the total genomic DNA that was isolated and used for library construction.

Given the current evidence regarding both the amino acid similarity between the genes that we have identified and the known genes cited in Figures 2 and 3 (see below), it is very likely that the gene BD5063 comes from either a *Pyrococcus* or *Thermococcus* species.

Figure 2 is a sequence alignment of the derived BD5088, parental protein sequences BD5063, BD5064 and BD5031, and the other known α-amylases isolated from *Thermococcus* and *Pyrococcus* species. It is clear that the α-amylase parental genes, and BD5088, are members of this highly related gene family.

Figure 2. Sequence Alignment of BD5088 Parents with Known Archaeal α-Amylases

```
                                          1                                                  50
                       BD5063     (1)  ------------------------------------MALEEGGLIMQA
                       BD5064     (1)  ---------MKPAKLLVFVLVVSILAGLYAQPAGAAKYLELEEGGVIMQA
                       BD5031     (1)  ---------MKKFVALFITMFFVVSMAVVAQPASAAKYSELEEGGVIMQA
                       BD5088     (1)  ----------------------------------MAKYSELEKGGVIMQA
  AAB87860 Thermococcus rt3       (1)  MSAKKLLALLFVLAVLVGVAVIPARVGIAPVSAGATSRPSLEEGGVIMQA
       AAC45663 P. furiosis       (1)  ----------MNIKKLTPLLTLLLFFIVLASPVSAAKYLELEEGGVIMQA
       AF240464_1 P. woesei       (1)  ----------MNIKKLTPLLTLLLFFIVLASPVSAAKYLELEEGGVIMQA
    BAA21130 Pyrococcus sp.       (1)  ---------MKKFVALLITMFFVVSMAAVAQPASAAKYSELEEGGVIMQA
Thermococcus hydrothermalis       (1)  -------------MARKVLVALLVFLVVLSVSAVPAKAETLENGGVIMQA
     Thermococcus profundus       (1)  ---------MGIRKTAAVLIVVLVALGISAVPAGAAKYSSLEDGGVIMQA
                  Consensus       (1)           M     L  LL LLV LGVLA PA AAKY ELEEGGVIMQA

                                          51                                                 100
                       BD5063    (13)  FYWDVPMGGIWWDTIAQKIPDWASAGISAIWIPPASKGMSGGYSMGYDPY
                       BD5064    (42)  FYWDVPSGGIWWDTIRQKIPEWYDAGISAIWIPPASKGMGGAYSMGYDPY
                       BD5031    (42)  FYWDVPGGGIWWDTIRSKIPEWYEAGISAIWIPPASKGMSGGYSMGYDPY
                       BD5088    (17)  FYWDVPSGGIWWDTIRQKIPEWYDAGISAIWIPPASKGMGGAYSMGYDPY
  AAB87860 Thermococcus rt3       (51)  FYWDVPAGGIWWDTIRSKIPDWASAGISAIWIPPASKGMSGAYSMGYDPY
       AAC45663 P. furiosis       (41)  FYWDVPGGGIWWDHIRSKIPEWYEAGISAIWLPPPSKGMSGGYSMGYDPY
       AF240464_1 P. woesei       (41)  FYWDVPRGGIWWDHIRSKIPEWYEAGISAIWLPPPSKGMSGGYSMGYDPY
    BAA21130 Pyrococcus sp.       (42)  FYWDVPAGGIWWDTIRSKIPEWYEAGISAIWIPPASKGMGGAYSMGYDPY
Thermococcus hydrothermalis       (38)  FYWDVPGGGIWWDTIAQKIPDWASAGISAIWIPPASKGMSGGYSMGYDPY
     Thermococcus profundus       (42)  FYWDVPGGAIWWDTIRSKIPEWYDAGISAIWIPPASKGMSGNSSMGYDPY
                  Consensus       (51)  FYWDVPGGGIWWDTIRSKIPEWYEAGISAIWIPPASKGMSGGYSMGYDPY

                                         101                                                 150
                       BD5063    (63)  DYFDLGEYYQKGTVETRFGSKQELINMINTAHAYGMKVIADIVINHRAGG
                       BD5064    (92)  DFFDLGEYDQKGTVETRFGSKQELVNMINTAHAYGIKVIADIVINHRAGG
                       BD5031    (92)  DFFDLGEYNQKGTIETRFGSKQELINMINTAHAYGIKVIADIVINHRAGG
                       BD5088    (67)  DFFDLGEYDQKGTVETRFGSKQELVNMINTAHAYGMKVIADIVINHRAGG
  AAB87860 Thermococcus rt3       (101) DFFDLGEYYQKGTVETRFGSKQELINMINTAHSYGIKVIADIVINHRAGG
       AAC45663 P. furiosis       (91)  DYFDLGEYYQKGTVETRFGSKEELVRLIQTAHAYGIKVIADVVINHRAGG
       AF240464_1 P. woesei       (91)  DYFDLGEYYQKGTVETRFGSKEELVRLIQTAHAYGIKVIADVVINHRAGG
    BAA21130 Pyrococcus sp.       (92)  DFFDLGEYNQKGTVETRFGSKQELINMINTAHAYGIKVIADIVINHRAGG
Thermococcus hydrothermalis       (88)  DFFDLGEYYQKGSVETRFGSKEELVNMINTAHAHNMKVIADIVINHRAGG
     Thermococcus profundus       (92)  DFFDLGEYYQKGTVETRFGSKQELVDMINTAHSYGIKVIADIVINHRAGG
                  Consensus       (101) DFFDLGEYYQKGTVETRFGSKQELVNMINTAHAYGIKVIADIVINHRAGG

                                         151                                                 200
                       BD5063    (113) DLEWNPFVNDYTWTDFSKVASGKYTANYLDFHPNELHAGDSGTFGGYPDI
                       BD5064    (142) DLEWNPFVNDYTWTDFSKVASGKYTANYLDFHPNEVKCCDEGTFGGFPDI
                       BD5031    (142) DLEWNPFVGDYTWTDFSKVASGKYTANYLDFHPNEVKCCDEGTFGGFPDI
                       BD5088    (117) DLEWNPFVNDYTWTDFSKVASGKYTANYLDFHPNELHAGDSGTFGGYPDI
  AAB87860 Thermococcus rt3       (151) DLEWNPFTNSYTWTDFSKVASGKYTANYLDFHPNEVKCCDEGTFGGFPDI
       AAC45663 P. furiosis       (141) DLEWNPFVGDYTWTDFSKVASGKYTANYLDFHPNELHCCDEGTFGGFPDI
       AF240464_1 P. woesei       (141) DLEWNPFVGDYTWTDFSKVASGKYTANYLDFHPNELHCCDEGTFGGFPDI
    BAA21130 Pyrococcus sp.       (142) DLEWNPFVGDYTWTDFSKVASGKYTANYLDFHPNEVKCCDEGTFGGFPDI
Thermococcus hydrothermalis       (138) DLEWNPFTNSYTWTDFSKVASGKYTANYLDFHPNELHAGDSGTFGGYPDI
     Thermococcus profundus       (142) DLEWNPFVGDYTWTDFSKVASGKYTANYEDFHPNEVKCCDEGTFGGFPDI
                  Consensus       (151) DLEWNPFVNDYTWTDFSKVASGKYTANYLDFHPNELKCCDEGTFGGFPDI

                                         201                                                 250
                       BD5063    (163) CHDKSWDQYWLWASQESYAAYLRSIGIDAWRFDYVKGYAPWVVRDWLNWW
                       BD5064    (192) AHEKSWDQYWLWASNESYAAYLRSIGVDAWRFDYVKGYGAWVVKDWLDWW
                       BD5031    (192) AHEKSWDQHWLWASDESYAAYLRSIGVDAWRFDYVKGYGAWVVKDWLNWW
                       BD5088    (167) CHDKSWDQYWLWASQESYAAYLRSIGIDAWRFDYVKGYAPWVVKDWLNWW
  AAB87860 Thermococcus rt3       (201) AHEKSWDQYWLWASQKSYAAYLRSIGIDAWRFDYVKGYGAWVVKDWLKWW
       AAC45663 P. furiosis       (191) CHHKEWDQYWLWKSNESYAAYLRSIGFDGWRFDYVKGYGAWVVRDWLNWW
       AF240464_1 P. woesei       (191) CHHKEWDQYWLWKSNESYAAYLRSIGFDGWRFDYVKGYGAWVVRDWLNWW
    BAA21130 Pyrococcus sp.       (192) AHEKEWDQHWLWASDESYAAYLRSIGVDAWRFDYVKGYGAWVVKDWLNWW
Thermococcus hydrothermalis       (188) CHDKSWDQHWLWASNESYAAYLRSIGIDAWRFDYVKGYAPWVVKNWLNRW
     Thermococcus profundus       (192) AHEKSWDQYWLWASDESYAAYLRSIGVDAWRFDYVKGYGAWVVKDWLSWW
                  Consensus       (201) CHEKSWDQYWLWASNESYAAYLRSIGIDAWRFDYVKGYGAWVVKDWLNWW
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000109

```
                                        251                                                  300
                     BD5063   (213)  GGWAVGEYWDTNVDAVLNWAYSSGAKVFDFALYYKMDEAFDNNNIPALVD
                     BD5064   (242)  GGWAVGEYWDTNVDALLNWAYSSDAKVFDFPLYYKMDAAFDNKNIPALVE
                     BD5031   (242)  GGWAVGEYWDTNVDALLNWAYSSGAKVFDFPLYYKMDEAFDNKNIPALVS
                     BD5088   (217)  GGWAVGEYWDTNVDAVLNWAYSSGAKVFDFALYYKMDEAFDNKNIPALVS
  AAB87860 Thermococcus rt3   (251)  A-LAVGEYWDTNVDALLNWAYSSGAKVFDFPLYYKMDEAFDNKNIPALVS
      AAC45663 P. furiosis    (241)  GGWAVGEYWDTNVDALLSWAYESGAKVFDFPLYYKMDEAFDNNNIPALVY
     AF240464_1 P. woesei     (241)  GGWAVGEYWDTNVDALLSWAYESGAKVFDFPLYYKMDEAFDNNNIPALVY
   BAA21130 Pyrococcus sp.    (242)  GGWAVGEYWDTNVDALLNWAYSSGAKVFDFPLYYKMDEAFDNTNIPALVD
Thermococcus hydrothermalis   (238)  GGWAVGEYWDTNVDALLSWAYDSGAKVFDFPLYYKMDEAFDNNNIPALVD
     Thermococcus profundus   (242)  GGWAVGEYWDTNVDALLSWAYDSNAKVFDFPLYYKMDEAFDNNNIPALVS
                  Consensus   (251)  GGWAVGEYWDTNVDALLNWAYSSGAKVFDFPLYYKMDEAFDNNNIPALV

                                        301                                                  350
                     BD5063   (263)  ALRYGQTVVSRDPFKAVTFVANHDTDIIWNKYPAYAFILTYEGQPTIFYR
                     BD5064   (292)  ALKNGGTVVSRDPFKAVTFVANHDTDIIWNKYPAYAFILTYEGQPTIFYR
                     BD5031   (292)  ALQNGQTVVSRDPFKAVTFVANHDTDIIWNKYLAYAFILTYEGQPVIFYR
                     BD5088   (267)  ALQNGQTVVSRDPFKAVTFVANHDTDIIWNKYPAYAFILTYEGQPTIFYR
  AAB87860 Thermococcus rt3   (300)  ALQNGQTVVSRDPFKAVTFVANHDTDIIWNKYPAYAFILTYEGQPVIFYR
      AAC45663 P. furiosis    (291)  ALQNGQTVVSRDPFKAVTFVANHDTDIIWNKYPAYAFILTYEGQPVIFYR
     AF240464_1 P. woesei     (291)  ALQNGQTVVSRDPFKAVTFVANHDTDIIWNKYPAYAFILTYEGQPVIFYR
   BAA21130 Pyrococcus sp.    (292)  ALQNGGTVVSRDPFKAVTFVANHDTDIIWNKYPAYAFILTYEGQPVIFYR
Thermococcus hydrothermalis   (288)  ALKNGGTVVSRDPFKAVTFVANHDTNIIWNKYPAYAFILTYEGQPAIFYR
     Thermococcus profundus   (292)  ALQNGGTVVSRDPFKAVTFVANHDTDIIWNKYPAYAFILTYEGQPVIFYR
                  Consensus   (301)  ALQNGQTVVSRDPFKAVTFVANHDTDIIWNKYPAYAFILTYEGQPVIFYR

                                        351                                                  400
                     BD5063   (313)  DYEEWLNKDKLKNLIWIHDNLAGGSTDDVYYDNDELIFVRN-GYGSKPGL
                     BD5064   (342)  DYEEWLNKDRLKNLIWIHDHLAGGSTDDVYYDNDELIFVRN-GYGDKPGL
                     BD5031   (342)  DYEEWLNKDRLNNLIWIHDHLAGGSTSIVYYDSDEMIFVRN-GYGSKPGL
                     BD5088   (317)  DYEEWLNKDKLKNLIWIHENLAGGSTDDVYYDNDELIFVRN-GYGDKPGL
  AAB87860 Thermococcus rt3   (350)  DYEEWLNKDRLKNLIWIHNNLAGGSTSIVYYDNDELIFVRN-GYGNKPGL
      AAC45663 P. furiosis    (341)  DFEEWLNKDKLINLIWIHDHLAGGSTTIVYYDNDELIFVRN-GDSRRPGL
     AF240464_1 P. woesei     (341)  DFEEWLNKDKLINLIWIHDHLAGGSTTIVYYDNDELIFVRN-GDSRRPGL
   BAA21130 Pyrococcus sp.    (342)  DYEEWLNKDKLNNLIWIHDHLAGGSTSIVYYDSDELIFVRN-GDSKRPGL
Thermococcus hydrothermalis   (338)  DYEEWLNKDRLRNLIWIHDHLAGGSTDIIYYDSDELIFVRN-GYGDKPGL
     Thermococcus profundus   (342)  DYEEWLNKDRLNNLIWIHEHLAGGSTKILYYDSDELIFEREGYGDKPGL
                  Consensus   (351)  DYEEWLNKDKLKNLIWIHDHLAGGSTSIVYYDNDELIFVRN GYG KPGL

                                        401                                                  450
                     BD5063   (362)  ITYINLGSSKAGRGWYYP-KFAGSCIHEYTGNLGGWVDKWVDSSGWVYLE
                     BD5064   (391)  ITYINLGSSKAGRWWYYP-KFAGACIHEYTGNLGGWVDKWVDSSGWVYLE
                     BD5031   (391)  ITYINLGSSKVGRGWYYP-KFAGACIHEYTGNLGGWVDKYVYSSGWVYFE
                     BD5088   (366)  ITYINLGSSKAGRWWYYP-KFAGACIHEYTGNLGGWVDKYVYSSGWVYLE
  AAB87860 Thermococcus rt3   (399)  ITYINLGSSKVGRWVYYP-KFAGSCIHEYTGNLGGWVDKYVGSNGWVYLE
      AAC45663 P. furiosis    (390)  ITYINLSPNWVGRWVYYP-KFAGACIHEYTGNLGGWVDKRVDSSGWVYLE
     AF240464_1 P. woesei     (390)  ITYINLSPNWVGRWVYYP-KFAGACIHEYTGNLGGWVDKRVDSSGWVYLE
   BAA21130 Pyrococcus sp.    (391)  ITYINLGSSKVGRWVYYP-KFAGACIHEYTGNLGGWVDKYVESSGWVYLE
Thermococcus hydrothermalis   (387)  ITYINLGSSKAGRWVYYP-KFAGSCIHEYTGNLGGWIDKWVDSSGRVYLE
     Thermococcus profundus   (392)  ITYINLGSDWTERWVNYGSKFAGYTIHEYTGNLGGWVDRYNQYDGWVKLT
                  Consensus   (401)  ITYINLGSSKVGRWVYVP KFAGACIHEYTGNLGGWVDKYVDSSGWVYLE

                                        451                 473
                     BD5063   (411)  AFAHDPANGQYGYSVWSYCGVG-
                     BD5064   (440)  AFAHDPANGYYGYSVWSYCGVG-
                     BD5031   (440)  AFAYDPANGQYGYSVWSYCGVG-
                     BD5088   (415)  AFAYDPANGQYGYSVWSYCGVG-
  AAB87860 Thermococcus rt3   (448)  AFAHDPAKGQYGYSVWSYCGVG-
      AAC45663 P. furiosis    (439)  AFPHDPANGYYGYSVWSYCGVG-
     AF240464_1 P. woesei     (439)  AFPHDPANGYYGYSVWSYCGVG-
   BAA21130 Pyrococcus sp.    (440)  AFAYDPASGQYGYTVWSYCGVG-
Thermococcus hydrothermalis   (436)  AFAHDPANGQYGYSVWSYCGVG-
     Thermococcus profundus   (442)  AFPYDPANGYYGYSIWSYAGVG-
                  Consensus   (451)  APAHDPANGQYGYSVWSYCGVG
```

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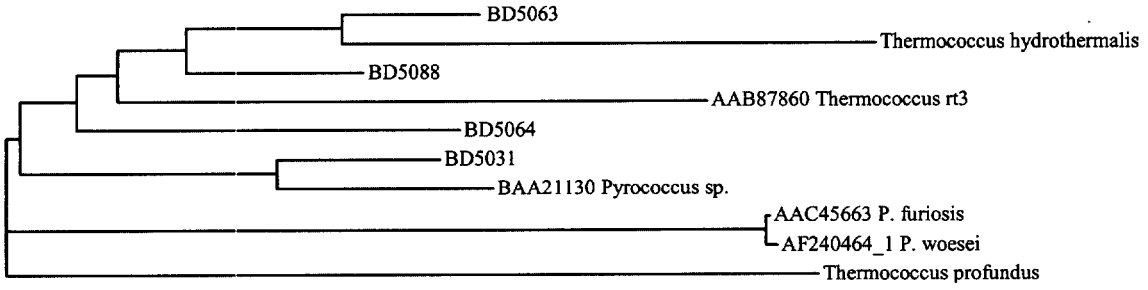
The % amino acid distance (similarity) between BD5088 and its three parental genes (Distances) is provided in Table 3.

Table 3. Comparison of BD5088 with its Parental Genes.

Amylase Source	BD5088	BD5031	BD5063	BD5064
BD5088	100	90	94	93
BD5031		100	89	90
BD5063			100	90
BD5064				100

Figure 3 graphically represents the familial relationship of the α-amylase sequences in the form of a dendogram or phylogenetic tree. It is clear that the α-amylase parental genes as well as BD5088 are members of this highly related archaeal gene family.

Figure 3. Phylogenetic Tree of the BD5088 Parental and Known Archaeal α-Amylases.



The % Distance amino acid homology between the known hyperthermophilic alpha amylases, BD5088, and its parent enzymes BD5063, BD5064 and BD5031 is presented in Table 4. The pairwise distances were calculated using the GCG (Genetics Computer Group, Madison, Wisconsin) Distances program using an uncorrected method.

Table 4. Homology Comparison of BD5088 With Native Hyperthermophilic α-Amylases

Amylase*	BD5063	BD5064	BD5031	BD5088	*Thermo Rt3*	P. furiosis	*P. woesei*	*Pyro-coccus sp.*	*T. hydro-thermalis*	*T. profundus*
BD5063	**100**	90.4	88.9	94.2	90.0	86.6	86.6	87.9	91.9	82.2
BD5064		**100**	89.4	92.4	87.8	85.0	85.0	89.1	86.0	85.0
BD5031			**100**	90.6	87.8	85.2	85.0	95.4	84.2	85.0
BD5088				**100**	89.7	85.8	85.8	89.9	89.7	84.6
Thermo Rt3					**100**	82.1	82.1	86.7	84.6	83.4
P. furiosis						**100**	99.7	85.7	82.5	81.1
P. woesei							**100**	85.7	82.5	80.9
Pyrococcus sp.								**100**	83.2	83.9
T. hydrothermalis									**100**	81.2
T. profundus										**100**

* All of the identified hyperthermophilic archaeal α-amylase sequences can be found in the scientific literature.

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9. Application Testing

Appendix 9

–Application Report–
Evaluation of BD5088 Alpha-Amylase Enzyme: Carbohydrate Profile and Residual Enzyme Levels

INTRODUCTION

Thermally stable α-amylases have been used by the Sweetener industry for many years as a processing aid for converting starches to sugars such as glucose and fructose. The α-amylase partially hydrolyzes the starch to reduce the viscosity of the gelatinized starch during primary liquefaction, and to produce dextrins of various sizes during secondary liquefaction. BD5088 is a thermally stable, protein engineered α-amylase enzyme that can operate at higher temperatures, lower pH and lower calcium levels than other commercial α-amylase enzymes. This report describes the carbohydrate profiles produced by BD5088, and commercially available protein engineered α-amylase enzymes derived from *Bacillus licheniformis* (Termamyl® LC) and *Bacillus stearothermophilus* (Termamyl SC), during liquefaction and the subsequent steps that are typically used in the Sweetener industry [1]. Measurement for residual BD5088 enzyme was also performed.

EXPERIMENTAL

Starch Slurry Preparation:
Corn starch slurries were prepared by suspending 75 lbs. of Cargill Pearl Food Starch 2700, batch # RL2428C (10.7% moisture content), or batch # RQ3288D (10.0% moisture content) in 14.0+/-0.2 gallons of deionized water at ambient temperature. For BD5088, calcium chloride dihydrate (3.2 g) was added to the starch slurry to give a calcium slurry concentration of 10 ppm, then the pH was adjusted to 4.8 using 1 N hydrochloric acid. These are typical conditions suggested by the BD5088 enzyme manufacturer. For Termamyl LC and Termamyl SC, calcium chloride dihydrate (6.4 g) was added to the starch slurries to give a calcium slurry concentration of 20 ppm, then the pH was adjusted to 5.8 using 1 N sodium hydroxide. These are typical conditions suggested by the Termamyl enzyme manufacturer [2]. The following enzyme formulations were then added to the starch slurries:

Enzyme Name:	Batch #:	Enzyme Activity:
BD5088	020619N	113 IAU/g [3]
Termamyl LC	AOP 00023	120 KNU/g [4]
Termamyl SC	AUP 00062	120 KNU/g [4]

After final pH adjustment to pH 4.8 for BD5088 or pH 5.8 for the Termamyl enzymes, the conductivity of the starch slurries was measured. The compositions of the starch slurries are given in Table 1.

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Primary Liquefaction of Starch:
The primary liquefaction of starch was performed using an Attec Continuous Starch Cooking pilot plant (Jet Cooker) [5]. The Jet Cooker pilot plant operated at a flow rate 0.3 gallons per minute. Steam was injected directly into the starch slurry to heat the slurry to 105°C and gelatinize the starch. The starch slurry was held at about 105°C for five minutes. During this time the BD5088 or other commercially available α-amylase enzymes hydrolyze the starch chains to reduce the viscosity of the gelatinized starch. Samples of the primary liquefaction hydrolyzate were collected in stainless steel thermos bottles and immediately transferred to 2800 ml Fernbach flasks in a hot (95-96°C) water bath. The primary liquefaction conditions are given in Table 1.

Table 1. Starch Slurry Recipe and Liquefaction Conditions Using Different α-Amylase Enzymes

Starch Slurry Composition						Primary Liquefaction Conditions		Secondary Liquefaction Conditions	
α-Amylase Enzyme Type (batch #)	Enzyme Amount (g/kg DS)	Dissolved Solids (%)	pH	Calcium Added (ppm)	Cond. (μS/cm)	Time (min)	Temp. (°C)	Time (hr)	Temp. (°C)
BD5088 (020619N)	0.50	35 (a)	4.8	10	387	5.0	105	2.0	95
Termamyl LC (AOP00023)	0.50	35 (a)	5.8	20	331	5.1	105	2.0	96
Termamyl SC (AUP00062)	0.50	35 (b)	5.8	20	460	5.0	106	2.0	96

DS = dissolved solids; Cond. = conductivity; Temp. = temperature;
(a) starch batch # RQ3288D; (b) starch batch # RL2428C

Secondary Liquefaction of Starch:
The secondary liquefaction of starch was performed by heating approximately 2400 g of the primary liquefaction hydrolyzate in a 2800 ml Fernbach flask at 95-96°C for two hours. Samples were removed at 15 minute time intervals and the reaction was quenched by lowering the pH to 3.0+/-0.3 with 1 N hydrochloric acid. The formation of reducing sugars was measured using Schoorl's method [6]. After two hours a sample of the secondary liquefaction hydrolyzate was also frozen and saved for carbohydrate analysis. The remaining hydrolyzate was cooled to 60°C using an ice bath and the pH was decreased to 4.3 to prepare the solution for saccharification. The secondary liquefaction conditions are given in Table 1.

Saccharification Procedure:
The secondary liquefaction hydrolyzate was transferred to a clean 2800 ml Fernbach flask, then the weight and dissolved solids (DS) level of the hydrolyzate were measured. Water was added to normalize the dissolved solids level from 31-32% DS to 30% DS and 1 N hydrochloric acid was added to reduce the pH to 4.3. Sigma *Aspergillus niger* amyloglucosidase (glucoamylase) enzyme, batch #85H02451 (0.53-0.61 ml), with an activity of 6000 Sigma units/g was added to give an enzyme concentration of 0.8 ml enzyme/kg DS. The mixture was placed in a water bath at 60°C for 44 hours

with periodic mixing. After 44 hours, the reaction was quenched by placing the Fernbach flask in a 95°C water bath for 20 minutes. A sample was frozen and saved for HPLC analysis. Purification of the remaining saccharification liquor was then immediately initiated.

Table 2. Saccharification Recipe and Conditions

α-Amylase Enzyme Used During Liquefaction	Saccharification Recipe			Saccharification Conditions	
	Dissolved (%) Solids	Enzyme Amount (ml/kg DS)	pH	Time (hr)	Temperature (°C)
BD5088	30.0	0.79	4.3	44	60
Termamyl LC	30.0	0.80	4.3	44	60
Termamyl SC	30.0	0.80	4.3	44	60

Purification Procedure [5]:
After saccharification, the sugars must be purified to remove contaminants such as insoluble solids, ash, protein, fatty acids, organic acids, and other byproducts formed from Maillard and base catalyzed reactions [10, 11]. Table 3 gives a list of the purification materials, the amounts used, and the temperatures of the columns during purification. Table 4 gives the composition of the glucose feed and the flow rate of the glucose through the granular activated carbon and the ion exchange resins. These materials are commonly used by the Sweetener industry to purify sugars. These purification conditions are typical for a plant producing fructose.

The hot saccharification liquor was filtered through a three inch layer (1000 ml) of diatomaceous earth (Celite 545) held in a 2000 ml coarse sintered glass filtration funnel. The filtered glucose was placed into a clean 2800 ml Fernbach flask in a 60°C water bath and pumped through a series of activated carbon and ion exchange resin columns at 10 ml/min. During purification samples were collected at regular time intervals and the dissolved solids level was measured. The sample with the highest dissolved solids level was frozen and saved for carbohydrate and residual BD5088 enzyme analysis.

Carbohydrate Analysis by HPLC:
The carbohydrate distribution and glucose purity were measured using high pressure liquid chromatography (HPLC) [7, 8]. Liquefaction hydrolyzate samples (1 part) were diluted with deionized water (6 parts) prior to HPLC analysis. Saccharification liquor and purified glucose samples (1 part) were diluted with deionized water (2 parts) prior to HPLC analysis. The equipment and chromatographic conditions are given below:

HPLC Equipment

Pump	HP1100 Quaternary High Pressure Mixing Pump
Autosampler	HP1100
Detector	Perkin Elmer Series 200 Refractive Index Detector
Column Heater	Jones Chromatography

HPLC Conditions

Columns	Bio-Rad Aminex Carbohydrate HPX-42A, 300 x 7.8mm Columns, (2) in series
Column Temperature	85° C
Mobile Phase	Milli-Q Water
Flow Rate	0.6 mL/min
Detection	Refractive Index
Integrator	PE/NELSON ACCESS*CHROM v1.9, with model 941 A/D interface
Injection Volume	20 µL

Table 3. Materials Used for the Purification of Glucose Solutions

Column #	Material Name	Batch #	Material Type	Volume (ml)	Temperature (°C)
	Celite 545	020577	DE	1000	~50
1	Calgon SGL 8x30	SO1917EJ5	GAC	1200	66
2	DOWEX® MONOSPHERE® 88 (H)	PI25012DC2	SAC	330	38
3	DOWEX MONOSPHERE 77	QD24012DF1	WBA	310	38
4	DOWEX MONOSPHERE 88 (H)	PI25012DC2	SAC	330	38
5	DOWEX MONOSPHERE 77	QD24012DF1	WBA	310	38
6	DOWEX MONOSPHERE 88 (H) DOWEX 22 (OH)	PI25012DC2 QD22012DG1	MR	40 60	32

DE = diatomaceous earth; GAC = granular activated carbon; SAC = strong acid cation exchange resin; WBA = weak base anion exchange resin; MR = mixed resin containing 40% SAC and 60% Type II strong base anion (SBA) exchange resin.

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Table 4. Column Purification Conditions for Glucose Solutions

α-Amylase Enzyme Used During Liquefaction	Glucose Feed		Purification Conditions						
	Amount (g)	Dissolved Solids (%)	Feed Rate (ml/min)	Flow Rates (BV/hour)					
				GAC Col. 1	SAC Col. 2	WBA Col. 3	SAC Col. 4	WBA Col. 5	MR Col. 6
BD5088	1422	33.1	10.0	0.5	1.8	1.9	1.8	1.9	6.0
Termamyl LC	1434	33.3	10.0	0.5	1.8	1.9	1.8	1.9	6.0
Termamyl SC	1286	31.7	10.0	0.5	1.8	1.9	1.8	1.9	6.0

BV = bed volumes; GAC = granular activated carbon; SAC = strong acid cation exchange resin; WBA = weak base anion exchange resin; MR = mixed resin containing 40% SAC and 60% Type II strong base anion (SBA) exchange resin; Col. = Column

Other Analytical Procedures:

The residual level of BD5088 enzyme in purified glucose was measured using a sandwich enzyme-linked immunosorbent assay (ELISA) [9]. The detection limit for this immunochemical assay is 2.4 ng/ml.

The dissolved solids (DS) level was measured using a Denver Instrument IR200 moisture analyzer.

The pH was measured using a Thermo Orion pH meter, model 230. The pH was calibrated using Fisher pH 4 and pH 7 buffer solutions.

The conductivity was measured using a Thermo Orion conductivity meter, model 115. The conductivity was calibrated using Myron L 442-300 and KCl-70 standard solutions.

RESULTS AND DISCUSSION

Thermally stable, protein engineered α-amylases have been used by the Sweetener industry for many years as a processing aid for converting starches to sugars such as glucose and fructose. The α-amylase partially hydrolyzes the starch to reduce the viscosity of the gelatinized starch during primary liquefaction, and to produce dextrins of various sizes during secondary liquefaction. These dextrins are then converted to sugars during saccharification using an amyloglucosidase enzyme. After saccharification, the sugars must be purified to remove contaminants such as insoluble solids, ash, protein, fatty acids, organic acids, and other byproducts formed from Maillard and base catalyzed reactions. Diatomaceous earth, activated carbon and ion exchange resins are used to remove the contaminants from the sugars [10, 11].

The carbohydrate profiles produced by BD5088 and two commercially available protein engineered α-amylase enzymes (Termamyl LC and Termamyl SC) were measured after liquefaction and the subsequent steps that are typically used in the Sweetener industry [1]. The residual level of active BD5088 after sugar purification was also measured.

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Liquefaction of Starch Using α-Amylase Enzymes

Table 1 gives the starch slurry recipes for the liquefaction of corn starch using BD5088 α-amylase. The starch slurry recipes for the liquefaction of corn starch using two commercially available α-amylase enzymes, Termamyl LC and Termamyl SC are also given in Table 1. Liquefaction using Termamyl LC and Termamyl SC are preferentially done around pH 5.8 with some calcium present (20 ppm was used). BD5088 shows good activity at a lower pH (4.8) and with less calcium present (10 ppm). The different conductivities of the starch slurries arise from the baseline conductivity of the two starch batches (350 μS/cm for batch # RL2428C; and 220 μS/cm for batch # RQ3288D) and from the different pH values.

The primary and secondary liquefaction conditions are also given in Table 1. These are typical conditions used in the Sweetener industry. During liquefaction partial hydrolysis of the starch occurs and reducing sugars are formed. The level of reducing sugars (dextrose equivalent) was measured using Schoorl's method [6]. The results are given in Table 5. The dextrose equivalent versus time curves (see Figure 1) show that the reducing sugar formation follows a similar course for all three α-amylase enzymes.

TABLE 5. REDUCING SUGAR FORMATION DURING LIQUEFACTION USING DIFFERENT α-AMYLASE ENZYMES

α-Amylase Used During Liquefaction →	BD5088	Termamyl LC	Termamyl SC
Time (hr)	**Dextrose Equivalent**		
0.25	4.4	4.8	3.7
0.50	6.2	5.7	5.9
0.75	7.8	7.2	8.1
1.00	9.0	9.0	9.7
1.25	10.1	9.3	11.5
1.50	10.9	9.6	12.6
1.75	11.7	9.9	13.8
2.00	12.5	11.1	15.5
Final Dissolved Solids (%):	31.7	30.9	31.4

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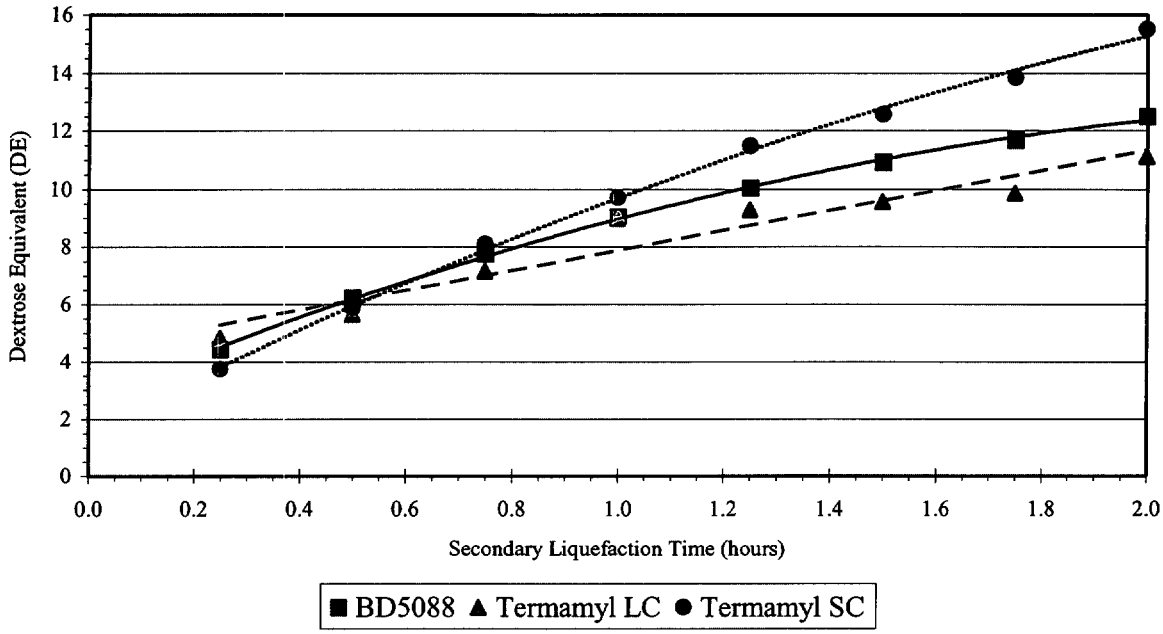
Figure 1.
Reducing Sugars Produced During Liquefaction Using Different α-Amylase Enzymes



The carbohydrate profiles for the secondary liquefaction hydrolyzate after two hours at 95°C were measured by high pressure liquid chromatography (HPLC) [8]. The chromatograms for the liquefaction hydrolyzates made from the three enzymes are shown in Figure 2. From the chromatograms it can be seen that all three α-amylase enzymes are substantially equivalent and hydrolyze the starch to produce the same oligosaccharides. The identity of the oligosaccharide peaks is indicated by the DP (degree of polymerization) labels, where: DP_2 identifies the di-saccharide peak; DP_3 identifies the tri-saccharide peak; DP_4 identifies the tetra-saccharide peak; etc. The relative amount of the different oligosaccharides varied according to the enzyme that was used. BD5088 produced an oligosaccharide profile that was intermediate between the oligosaccharide profiles that were produced by Termamyl LC and Termamyl SC.

Saccharification of Hydrolyzate

After liquefaction the partially hydrolyzed starch was converted to glucose using an amyloglucosidase enzyme during the saccharification step. The glucose yield can be affected by incomplete hydrolysis or by-product formation during the liquefaction step. The secondary liquefaction hydrolyzates that were made using the three different α-amylase enzymes were saccharified using a readily available amyloglucosidase enzyme (Sigma *Aspergillus niger*). The saccharification recipe and conditions are given in Table 2. These are typical conditions used in the Sweetener industry. Water was initially added to the hydrolyzate to reduce the dissolved solids level from 31-32% DS to 30% DS. The glucose yield was measured by HPLC after 44 hours of saccharification at 60°C. The results are given in Table 6.

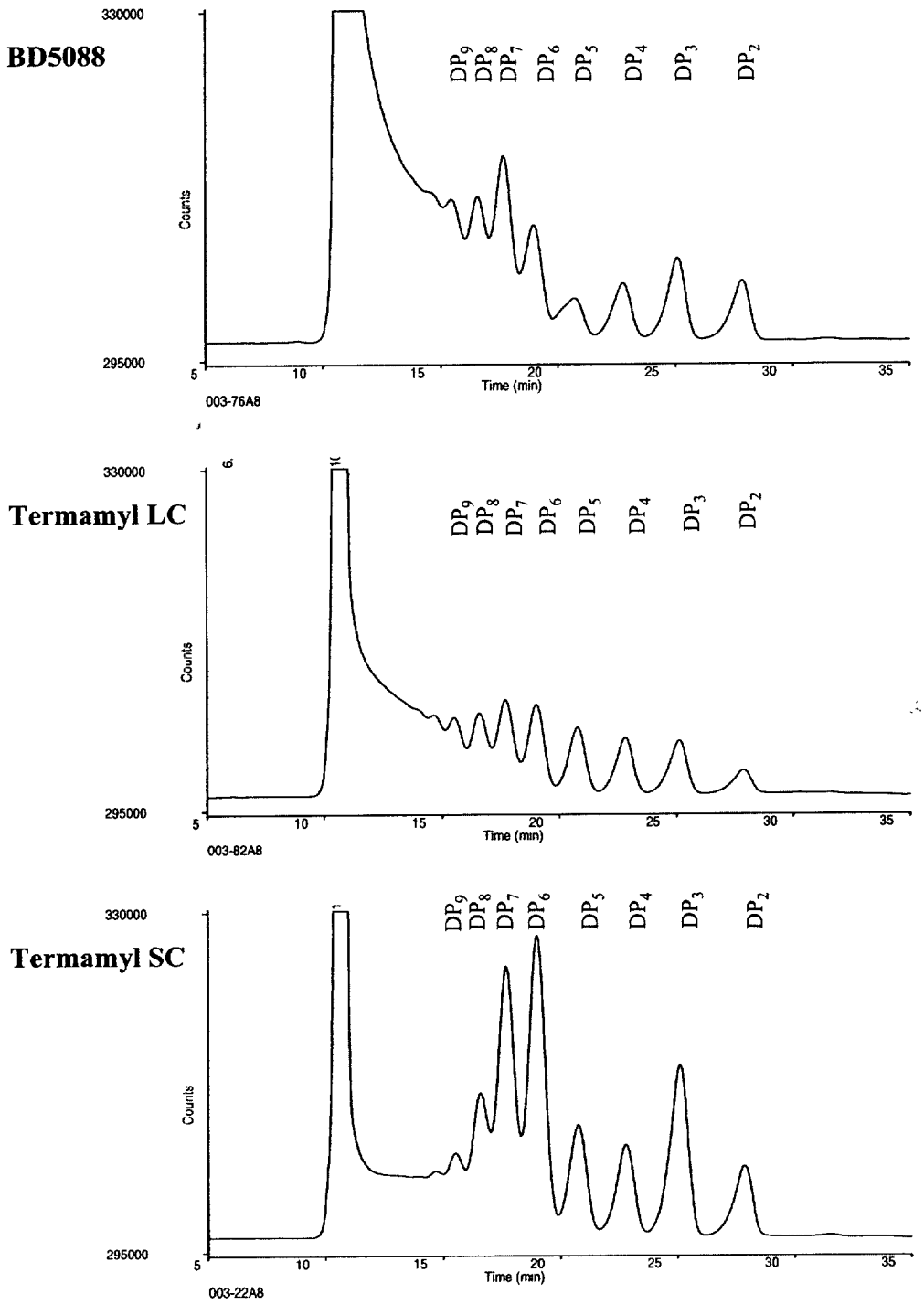
Table 6. Carbohydrate Distribution after 44 Hours of Saccharification at 60°C.

α-Amylase Used During Liquefaction	Final Dissolved Solids (%)	Carbohydrate Distribution (w/w %)			
		Glucose	DP_2	DP_3	DP_4+
BD5088	33.5	95.7	2.5	1.2	0.7
Termamyl LC	33.6	95.9	2.6	0.7	0.8
Termamyl SC	32.7	95.5	2.6	0.3	1.7
Typical Industry Values [12]:	33	95 - 96	2.0 - 4.0	0.4 - 0.8	0.6 - 1.0

DP = degree of polymerization, DP_2 = di-saccharides,
DP_3 = tri-saccharide, DP_4+ = tetra- and larger saccharides

The glucose yield (95.7%) after saccharification using the liquefaction hydrolyzate made from BD5088 was identical to the glucose yields (95.5%-95.9%) using the hydrolyzates made from two commercially available enzymes, and to the typical glucose yields (95-96%) obtained in the Sweetener industry [12]. The carbohydrate distribution also showed no significant differences. Thus, the saccharification liquors produced using hydrolyzate from all three enzymes were substantially equivalent.

Figure 2.
Carbohydrate Profile after 2 Hours of Secondary Liquefaction at 95-96°C Using Different α-Amylase Enzymes



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Purification of Glucose Solutions

Diatomaceous earth, activated carbon and ion exchange resins are used to purify sugars [10,11]. The purification materials that were used are given in Table 3. These materials are commonly used by the Sweetener industry to purify sugars. The purification conditions that were used are given in Table 4. These purification conditions are typical for a plant producing glucose, and then converting it to fructose. The saccharification liquors produced from liquefaction hydrolyzate made using BD5088 and two commercially available α-amylase enzymes were filtered through diatomaceous earth, then the glucose solutions were pumped through a series of columns containing granular activated carbon and ion exchange resins. Effluent samples from the last column (mixed resin) were collected at regular time intervals and the dissolved solids level of the purified glucose fractions was measured. The profile for the dissolved solids versus time is shown in Figure 2. These three profiles have a similar appearance with the minor differences being attributed to the slightly different amounts of glucose fed to the columns.

The sample fraction that contained the highest dissolved solids content for each experiment was analyzed by HPLC for glucose purity and carbohydrate distribution. The results are given in Table 7. The glucose purity after purification was identical using saccharification liquor feeds prepared from liquefaction hydrolyzate made using BD5088, Termamyl LC and Termamyl SC. The carbohydrate distributions also showed no significant differences. Fructose likely arose from isomerization of a small amount of the glucose as it passed through the mixed resin column. New ion exchange resins were used for each experiment to eliminate the chance of cross contamination, and new strong base anion resin in a mixed resin can cause isomerization of glucose to fructose. Thus, the purified glucose made from saccharification liquors produced using hydrolyzate made from all three enzymes were substantially equivalent.

Figure 3.
Glucose Concentration Profile During Purification of Glucose Containing Different α-Amylase Enzymes

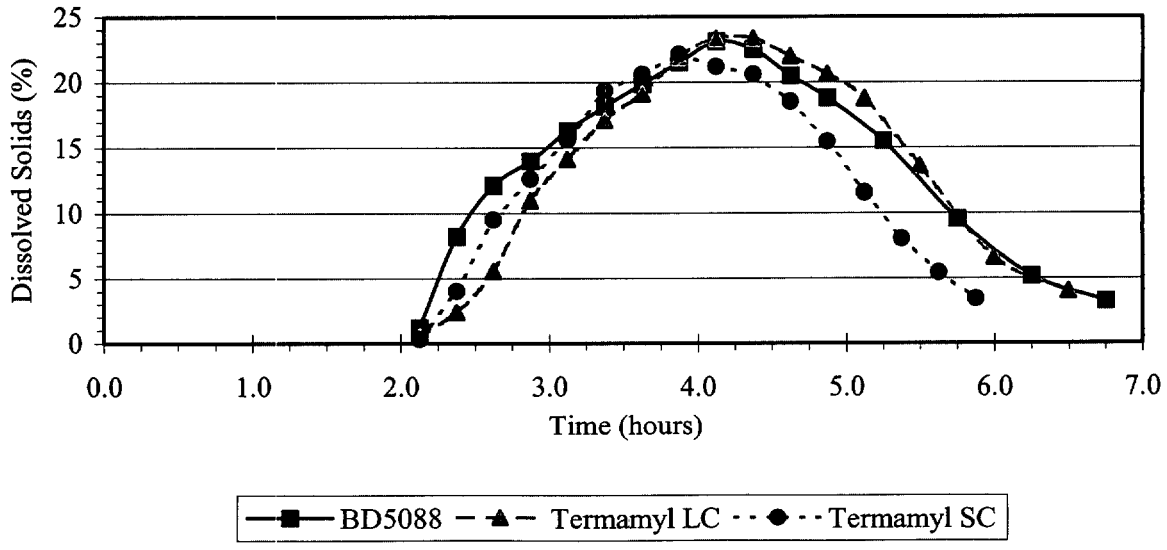

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Table 7. Carbohydrate Distribution after Purification

α-Amylase Used During Liquefaction	Collection Time (hr)	Dissolved Solids (%)	Carbohydrate Distribution (w/w %)				
			Fructose	Glucose	DP_2	DP_3	DP_4+
BD5088	4.1	23.1	1.4	98.1	0.4	0.0	0.1
Termamyl LC	4.4	23.3	1.0	97.8	1.2	0.0	0.0
Termamyl SC	3.9	22.1	1.3	97.8	0.9	0.0	0.1

DP = degree of polymerization, DP_2 = di-saccharides,
DP_3 = tri-saccharides, DP_4+ = tetra- and larger saccharides

RESIDUAL BD5088 LEVEL IN PURIFIED GLUCOSE

During the liquefaction, saccharification and purification steps that are used to produce sugars from starches, enzymes become inactivated and are removed from the sugar solution. The residual level of BD5088 α-amylase enzyme in the purified glucose sample was measured using a sandwich enzyme-linked immunosorbent assay (ELISA) method [9]. BD5088 was not detected in the purified glucose sample at a detection limit of 2.4 ng/ml (ppb), which is equivalent to no BD5088 enzyme detected at a level of 9.5 μg/kg on a 100% dissolved solids basis.

CONCLUSIONS

The following conclusions were drawn from this study about the carbohydrate profile produced by BD5088 compared to two commercially available α-amylase enzymes:

- Reducing sugar formation followed a similar course for all three enzymes during liquefaction.
- The same oligosaccharides were produced by all three enzymes during liquefaction.
- The relative amount of each oligosaccharide varies slightly depending on the enzyme that was used during liquefaction. BD5088 produced an oligosaccharide profile that was intermediate between Termamyl LC and Termamyl SC.
- The glucose yields (95.7+/-0.2%) and the carbohydrate profiles were equivalent after saccharification using the liquefaction hydrolyzates made from each of the three enzymes.
- The glucose purity (97.9+/-0.2%) and the carbohydrate profiles were equivalent after purification of the saccharification liquors produced from liquefaction hydrolyzates made from each of the three enzymes.
- No BD5088 enzyme was detected in the purified glucose solution at a detection limit of 2.4 ng/ml (ppb), which is equivalent to no BD5088 enzyme detected at a level of 9.5 μg/kg on a 100% dissolved solids basis.

In summary, it was found that the sugar profiles after liquefaction, saccharification and purification are substantially equivalent for BD5088 and α-amylase enzymes currently used in commerce.

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10. Safety Article

Pages 000127 - 000146 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

11. Pathogenicity Review

STANFORD UNIVERSITY SCHOOL OF MEDICINE
DEPARTMENT OF MICROBIOLOGY & IMMUNOLOGY
SHERMAN FAIRCHILD SCIENCE BUILDING
STANFORD, CALIFORNIA 94305-5124

FAX: 650.725.6757

(b)(6)

4/3/02

Phone: 650/723-9187
Fax: 650/725-7282
falkow@stanford.edu

March 21, 2002

John W. Davis
1803 Building
Toxicology & Environmental Research Laboratory
The Dow Chemical Company
Midland MI 48674

Dear John:

I'm writing to provide you with my final comments about the study performed for you on the "Evaluation of Pathogenicity of *Pseudomonas fluorescens*, Biovar 1, strain MB101, in BALB/C Mice" by BRT-Burleson Research Technologies, Inc. We discussed by telephone today some of the minor corrections to be made to the report like the date the mice were received at Burleson. I would like to reiterate the section of the report devoted to Statistical Analysis (section 3.6) should be more clearly stated. It would also be useful if you could determine from the Burleson scientists if the API20 diagnostic strip identification was specific for *Pseudomona fluorescens* or if the identification code might include other pseudomonad species as well.

None of these points are of great importance except to make the final report as informative as possible nor do they impact the fundamental conclusion of this study, which there is absolutely no evidence of any intrinsic pathogenicity of the *P. fluorescens* Biovar 1, strain MB101 in BALB/C mice. Indeed, the data, much of which is summarized in Table 2, demonstrate that the numbers of *P. fluorescens* found in the tissue do not significantly exceed those for background *Pseudomonas* species cultured from non-infected control animals. Moreover, the data show that at least 99.99999% of the bacteria fed to the animals cannot be recovered from the intestinal tract of the animals after 48 hours. A few pseudomonas colonies were recovered from internal organs but this was not significant different from the pattern seen in uninfected controls. I point out it is quite common to find such low numbers of bacteria in the internal organisms of perfectly normal animals. In my experience this is parallel to that which we see when challenging animals with known avirulent bacteria or with avirulent mutants of pathogenic bacteria. Thus, there is no sign whatsoever of infection or even transient colonization following challenge with over 600 million bacteria of the MB101 strain of *P. fluorescens*.

When Dow first asked me for my help and advice, we agreed the most important objective was to devise a straightforward way to test any potential intrinsic pathogenicity in the *P. fluorescens* strain you propose to use for commercial development. The studies you authorized and carried out are the same as those used in my laboratory to measure the pathogenicity of pathogenic enteric bacteria. Using this criteria, it is my professional judgment the strain of *P. fluorescens* you propose to utilize is non-pathogenic and does not represent an infectious threat of any kind to humans who might come into contact with these bacteria.

I have enjoyed working with you and your colleagues. I have been impressed with the care taken by you and your associates to insure that this microbe you plan to use represents no health risk to your workers or to those who may use it or products derived from it in the future.

Sincerely yours,

(b)(6)

Stanley Falkow, Ph.D.
Professor of Microbiology and Immunology

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12. Histopathology Review

MEMORANDUM

RECEIVED
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8/12/02

To: David S. Hanselman, PhD
Director of Regulatory Affairs
Environmental Health and Safety
Innovase LLC
5501 Oberlin Drive
San Diego, CA 92121

Timothy D. Landry, PhD, DABT
Toxicology Consultant
Toxicology and Environmental Research and Consulting
The Dow Chemical Company
Building 1803
Midland, Michigan 48674

From: Jack R. Harkema, DVM, PhD, Diplomate ACVP (b)(6) 8/12/02
Professor of Comparative Pathology
Michigan State University

Subject: Histopathology Review of Nasal and Pulmonary Tissues from Fischer 344 Rats in BD5088 Alpha-amylase 90-Day Oral Gavage (File Number 001232) and 14-Day Dietary (File Number 021037) Toxicology Studies

INTRODUCTION: I microscopically examined nasal and pulmonary tissues (four sections of nose and five sections of lung) from all the male rats in the medium and high dose groups (300 and 1000 mg/kg, respectively) and from at least half of the male rats in the control and low dose groups (0 and 100 mg/kg, respectively) in the 90-Day Oral Gavage Study. I also examined similar nasal and pulmonary tissues from all the male and female rats in the 300 mg/kg group and at least half of the male and female rats in the 0 mg/kg (control) group of the 14-Day Dietary Study.

HISTOPATHOLOGY: RESULTS OF 90-DAY ORAL GAVAGE TOXICOLOGY STUDY

Most of the examined rats in the 90-Day Oral Gavage Toxicology Study that were gavaged with 100, 300, or 1000 mg/kg of the compound had nasal and/or pulmonary lesions related to the treatment. No significant lesions were found in the control rats (0 mg/kg). The principal airway lesions in the compound-treated rats were a chronic active rhinitis, bronchiolitis, and alveolitis. Individual severity of the lesions ranged from minimal to marked. However, animals in the high-dose group had overall more severe lesions than those in the low or medium dose groups.

The compound-induced rhinitis was bilateral and characterized by a mixed inflammatory cell influx in the nasal mucosa consisting of neutrophils and mononuclear cells (lymphocytes and plasma cells). The cellular influx was evident to varying degrees in all four sections (T1 – T4, proximal to distal respectively) and was present in mucosal tissues lined by transitional, respiratory or olfactory epithelium depending on the intranasal site.

The rhinitis was most conspicuous and consistent in the mucosa lining the lateral meatus in T1, the mid septum, lateral meatus, and proximal aspect of the third ethmoturbinate in T2, the mid and ventral septum, fourth, fifth, and sixth ethmoturbinates, and nasopharyngeal meatus in T3, and the nasopharyngeal meatus in T4. Often in the most severely affected nasal tissues a mucopurulent (catarrhal) exudate was present in some of the nasal airways (meatuses).

Associated with the inflammatory response were alterations in the respiratory surface epithelium consisting of mucous cell hyperplasia/hypertrophy with marked increases in the amount of stored mucosubstances in the respiratory epithelium lining the proximal septum and medial aspect of the nasoturbinates (T2) and the nasopharyngeal meatus (T3 and T4). Mucous cell metaplasia (appearance of mucous cells in areas normally containing few or no cells of this phenotype) was conspicuous in the mid septum of T2 and in the maxillary sinus (T3). Lymphoid hyperplasia was often present in the nasal associated lymphoid tissue (NALT) in T3 and T4 of affected animals. Mild to moderate hyperplasia/hypertophy of the transitional epithelium lining the proximal lateral meatus (T1) was occasionally present in rats with the most severe rhinitis.

The compound-induced lesions in the lung were characterized by a similar mixed inflammatory cell influx of neutrophils and mononuclear cells in affected axial, pre-terminal and terminal bronchioles (chronic active bronchiolitis). A mucopurulent exudate was also present in some of the more severely affected conducting airways. Mucous cell metaplasia of the surface epithelium lining the conducting airways was commonly associated with this airway inflammation. Lymphoid hyperplasia of the bronchial associated lymphoid tissue (BALT) was also a common feature in affected axial airways. Focal areas of centriacinar alveolitis often accompanied the bronchiolitis and consisted of neutrophils, monocytes, and increased numbers of alveolar macrophages. In more severely affected areas, type II cell hyperplasia, hemorrhage and edema were also present in the alveolar parenchyma.

HISTOPATHOLOGY: RESULTS OF 14-DAY DIETARY TOXICOLOGY STUDY

No compound-related lesions were present in either nasal or pulmonary tissues examined from male or female rats in the 300 mg/kg group. No nasal or pulmonary lesions were present in male or female control rats.

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CONCLUSIONS:

The nasal and pulmonary lesions in the compound-treated rats in the 90-Day Oral Gavage Study were airway associated and likely due to aspiration of the compound during or

after gavage. The character of the inflammatory cell response and the airway epithelial alterations are remarkably similar to those previously reported in rats that were experimentally exposed by inhalation or intra-airway instillation to bacterial endotoxin (1-4).

I concur with the study pathologist's interpretation of the airway lesions in the 90-Day Oral Gavage Study. The lack of similar airway lesions in animals fed the compound in the 14-Day Dietary Study additionally supports the idea that the affected rats in the 90-Day Oral Gavage Study aspirated the compound during or after the gavage procedure.

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13. LPS Background

Appendix 13

Background Discussion on Endotoxin

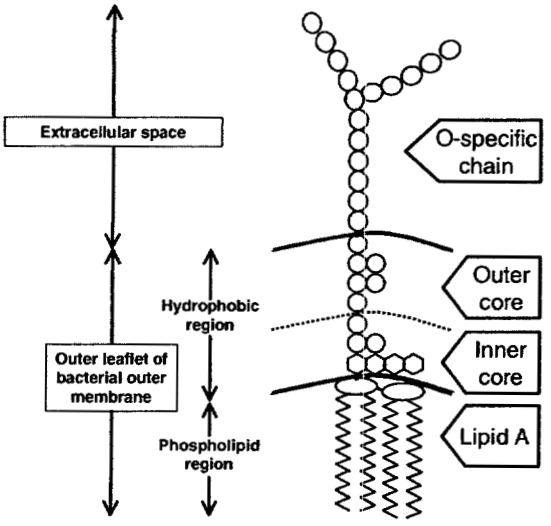

Figure 1. Schematic representation of the structure of LPS.

Endotoxin (lipopolysaccharide, LPS) is a component of the cell walls of Gram negative bacteria. Exposure to small doses of endotoxin is common and episodic and, in large part, occurs without serious adverse health effects. Under some circumstances, however, endotoxin can exacerbate tissue injury from other agents. In addition, exposure to large doses of endotoxin is associated with adverse consequences. The initiating event is an inflammatory response. Considerable attention has been given to LPS because contamination of materials with intended parenteral administration can cause effects associated with the potential of LPS to cause inflammation. Among the consequences are fever (Heumann et al. 1992, Nichols, et al., 1988), alterations in white blood cell count (Elin et al., 1981), changes in ventilation (Burrell, 1994), and circulatory disturbances. Environmental endotoxin comprises lipopolysaccharides, phospholipid, and proteins of bacterial membrane origin. Rietschel (1994) has reviewed the structure and function of endotoxin. Biological activity is associated with the lipopolysaccharide (LPS) component. The average molecular weight of LPS is 8,000; structure is provided in Figure 1. The sources of LPS, Gram negative bacteria, are widespread in nature. The most common potential source of LPS exposure is the usually harmless, enteric bacteria of the species *Escherichia* and *Salmonella* that colonize the lower part of the gastrointestinal tract in humans. A common pathogenic mode of exposure is from a Gram negative-bacterial infection. Common sources of exposure include agricultural and other occupational processes as well as domestic environments.

Oral gavage administration of BD5088 enzyme preparation resulted in respiratory tract inflammation in some rats. Contamination and direct contact due to aspiration was considered to cause the inflammation. Administration of BD5088 enzyme preparation in feed to rats resulted in no effects on the respiratory tract. Dr. Jack Harkema provided an independent review of the slides from the 2-week dietary and 13-week oral gavage studies. His report (Appendix 12) describes the effects of LPS on the respiratory tract and confirmed the interpretation that aspiration of the enzyme preparation caused the respiratory inflammation. Harkema saw no such effects in rats provided the enzyme preparation in feed.

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Appendix 14

Evaluation of Potential for Production Strain Pathogenicity: A Literature Survey

SUMMARY

The potential for pathogenicity and the potential for toxin production must be primary considerations when evaluating the safety of a production strain of organism used for the manufacture of a microbial enzyme preparation (Pariza and Johnson, 2001). As part of the overall evaluation of the safety of the production organism, *Pseudomonas fluorescens*, Biovar I, strain DC88, used to produce alpha-amylase BD5088, the scientific literature was reviewed to identify publications that might provide insight into the potential pathogenicity of *P. fluorescens* and its potential to produce toxins; *i.e.*, its potential for toxigenicity. Three separate literature searches were conducted. The objectives of the first two searches were similar and focused on the toxigenicity and pathogenicity of *P. fluorescens*. The objective of the third search was to evaluate the potential for toxin production by any *Pseudomonas* species.

The three literature searches resulted in the identification of approximately 800 potentially relevant reports. The majority of the reports were not directly relevant to the objectives of the searches and contained little information related to the toxigenicity or pathogenicity of *P. fluorescens*. However, a small subset of the reports was found to contain information relevant to the potential for causing adverse effects in humans. The information covered by these articles could be grouped into the following categories:

1) Evaluation of the pathogenicity of *P. fluorescens* in animal models.

2) Role of *P. fluorescens* infections in individuals with compromised immune systems.

3) *In vitro* cytotoxic effects of pigments (pyoverdine) purified from *P. fluorescens*.

4) Growth of *P. fluorescens* in blood products or medical devices.

A brief summary of the references from these four major categories, as well as several of the more relevant studies related to the safety of *P. fluorescens,* are presented in this review. No reports were found that claimed to provide evidence that *P. fluorescens* is a pathogen to healthy humans or implicating this microorganism in the production of any substances which would be toxic to humans by the oral route. Based on the results of the literature review, *P. fluorescens* was confirmed as generally nontoxigenic and nonpathogenic. Therefore, food ingredients produced from the production strain, if made according to Good manufacturing Practice, should be safe for human consumption (IFBC, 1990; Pariza and Johnson, 2001).

General Characteristics of Pseudomonas

Pseudomonas species are ubiquitous saprophytes, with low virulence, and are primarily opportunistic pathogens in hosts with low general or local resistance (Palleroni 1990). The American Type Culture Collection (ATCC) has designated strains of *P. fluorescens* under Biosafety Level 1 (BSL-1) which is defined as "...having no known potential to cause disease in humans or animals."

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Pseudomonas species can grow on a range of very simple organic substrates and they can remain viable for long periods of time in a wide variety of habitats. *P. fluorescens* is widely distributed in the environment (Bradbury, 1986) and has been isolated from soils, water, plants, animals, the hospital environment, and human clinical specimens. *P. fluorescens* is also a normal inhabitant of the plant rhizosphere or phyllosphere environment (Trevors *et al.*, 1990). *P. fluorescens* has occasionally been associated with human infection but the inability of most strains to grow at normal human body temperature (Palleroni, 1992a) restricts invasion and subsequent disease promotion. Although growth of *P. fluorescens* is restricted at higher temperatures this organism has the ability to grow at 4 °C (Gilardi, 1991).

Characteristics of Pathogenic Organisms

Microorganisms cause disease by two mechanisms: 1) invasion of tissue (*e.g.*, infection) and 2) production of toxins. Pathogenic microorganisms produce substances that are directly or indirectly toxic to host cells (Finaly and Falkow, 1997). However, an organism may be present in a host, and culturable from tissues without causing the disease. Exotoxins are secreted microbial products that damage or kill host cells at extremely low concentrations. Exotoxins play a key role in the pathogenicity of microorganisms and often the toxin alone can cause many of the clinical signs of a disease (Davis *et al.* 1973).

Pathogenicity is a function of the ability of the microorganism to invade the cell and the manner in which the infected hosts respond to the microbial invasion. Although a healthy human is populated with a variety of bacteria, even some that can cause disease, most individuals have an array of natural mechanisms of antibacterial defense (Davis *et al.* 1973). Nonpathogenic organisms are bacterial species that are incapable of penetrating natural defenses of a host, while pathogenic organisms can overcome the natural defenses of the host and cause disease. The degree of pathogenicity for microorganisms may vary over a wide range.

Toxigenic potential refers to "synthesis by the production strain of toxins that are active via the oral route" (Falkow, 1997; Pariza and Johnson, 2001). Pathogenic refers to the ability of an organism to cause disease in a healthy individual (as compared to a immuno-compromised host). It is reasonable to conclude that if studies specifically designed to demonstrate toxigenicity and pathogenicity of a strain *in vitro* and/or *in vivo* are negative, then that strain can be used safely in the production of food-grade enzyme preparations.

Methods

Three separate literature searches were conducted. The first search was performed in March of 1998 Nerac, Inc. Specifically, the search focused on *Pseudomonas fluorescens* using the key words: health, toxicity and safety. This search yielded 53 references.

A more extensive second search was conducted, where over 25 data bases were searched, including The Dow Chemical Company Technology Reports 1937-present, Chemical Abstracts 1947-present, Agricola 1970-present, CABA 1973-present, Biosis 1969-present, Medline 1958-present, Toxline 1965-2000, Toxlit 1965-present, Embase 1974-present, Nioshtic 1973-1998, SciSearch 1974-present. CCRIS, DART/ETIC, EMIC, Gene-Tox, and IRIS were searched through TOXNET, and the following databases available through the Chemical Information System: AQUIRE - Aquatic Information Retrieval, BIODEG - Biodegradation of Substances in

the Environment, BIOLOG - Biodegradation Literature References, BRS - Biennial Reporting System, CCRIS - Chemical Carcinogenesis Research Information System, CERCLIS - CERCLA Information, CHRIS - Chemical Hazard Response Information System, DATALOG, DOCKET - US EPA Civil Enforcement Docket, ENVIROFATE - Environmental Fate, FINDS - Facilities Index Data System, GIABS - Gastrointestinal Absorption, HSDB - Hazardous Substances Data Bank, ISHOW - Information System for Hazardous Organics in Water, MEDLINE Toxicology Subset, MSDSs (BAKER and MALLIN), NIOSHTIC® Subset, PHYTOTOX - Plant Toxicity, RCRIS - Resource Conservation and Recovery Information System, RTECS® - Registry of Toxic Effects of Chemical Substances, SANSS - Structure and Nomenclature Search System, TERRETOX - Terrestrial Toxicity, TRI - Toxics Release Inventory, TSCAINV - Toxic Substances Control Act Inventory, TSCAPP - TSCA Plant and Production, TSCATS - Toxic Substances Control Act Test Submissions.

The search strategy was conducted using the following key words or phrases: *Pseudomonas fluorescens* AND (toxic* or pathogen* or cytotox* or genotox* or mutagen*) and (human* or man or men or women or woman or child* or infant* or worker* or employee* or patient. Approximately 117 references were retrieved.

A third search, distinct from the first two, focused on toxin production in all *Pseudomonas* species. In order to limit the number of references to a manageable number, fewer databases were searched, including (Chemical Abstracts, Agricola, CABA, Biosis, Medline, Embase and NIOSHTIC) and the key words or phrases included pseudomonas and (toxic* or cytotox* or genotox*) and (human* or man or men or woman or women or child* or infant* or worker* or employee*).

This limited strategy still produced a list of over 15,000 citations. To further limit the number of citations selected references were eliminated and the terms aeruginosa or tumor* or sarcoma* or neoplasm* or cancer* or chimera or exotoxin A were negated (only in CABA, Biosis, Medline, Embase and Nioshtic). Approximately 629 references were retrieved using this approach.

Results of Literature Surveys

Searches 1 & 2: Toxigenicity or Pathogenicity of *Pseudomonas fluorescens*

In 1991 an extended review of the literature was conducted. This search was focused primarily on whether *P. fluorescens* biotype A (*i.e.* Biovar 1) could cause toxic effects on plants or animal species. The review concluded that "no data demonstrating that the organism is toxic to mammals was found." In March 1998, the original search was updated. The MedLine database was searched for information on *Pseudomonas fluorescens* using the search string "tox*" which include variation of the word toxicity (toxic, toxicology etc.). The majority of the citations retrieved from this search described toxic effects upon *P. fluorescens*, or uses of *P. fluorescens* to detect toxic conditions as in bioassay applications. No new information was found that implicated *P. fluorescens* in plant or animal pathology.

The second search, which focused on the potential toxicity or pathogenicity of *P. fluorescens,* was conducted in October of 2001. This search resulted in 117 citations, but 65% of these were not directly applicable to evaluating the effect of *P. fluorescens* on humans. Approximately one-third of these non-applicable references focused on the use of *P. fluorescens* to measure toxicity

of chemical agents while the remaining two-thirds of the references addressed the role of this microorganism as the normal microbial component of plants/animals/soil/animals/water/food.

Forty of the 117 citations/abstracts contained information relevant for evaluating the potential toxigenicity or pathogenicity of *P. fluorescens* to humans. The information covered in these references was grouped into four major categories:

1) Evaluation of the pathogenicity of *P. fluorescens* in animal models.

2) Role of *P. fluorescens* infections in individuals where the immune systems had been compromised (*e.g.,* role as an opportunistic pathogen)

3) *In vitro* cytotoxic effects of a pigment (pyoverdine) purified from *P. fluorescens*

4) Growth of *P. fluorescens* in blood products or medical devices.

A brief synopsis of the references from these four major categories is presented below. No reports were found that provided evidence that *P. fluorescens* is a pathogen to healthy humans or implicating this microorganism in the production of any substances which would be toxic to humans by the oral route.

Evaluation of the Pathogenicity of *P. fluorescens* in Animal Models

The U. S. Environmental Protection Agency (EPA) conducted two *in vivo* studies to evaluate the possible health concerns associated with the use of *P. fluorescens* as a microbial pest control agent (George *et al.*, 1999, George *et al.*, 2000). In the first study, male C3H/HeJ mice were administered intranasally with ~10^7 CFU (colony forming units) of *P. fluorescens* per mouse (George *et al.*, 1999). Clearance of the dosed bacterial strains from lungs, small intestine, large intestine, cecum, mesenteric lymph nodes (MLN), spleen, and liver were evaluated. Bacterial clearance requires an intact and functional immune system that incorporates a cascade of immune responses. In this animal model bacterial clearance provides an indication of the interaction between the potential pathogenicity of the invading microorganism and the total host immune capability.

The results of the study by George and coworkers (1999) demonstrated that *P. fluorescens* was eliminated from the lungs, cecum, small and large intestine by 2 day post-treatment. *P. fluorescens* was detected in the liver and MLN 3 hr after treatment but had disappeared completely from these tissues within 2 days of treatment. No mortality in the mice was noted at bacterial concentrations as high as 5.0 X 10^8 CFU/mouse although some mortality was observed at excessively high (~10^9/mouse) bacterial concentrations.

In the second study, male CD-1 mice were treated perorally with average dose of 1.78×10^8 CFU of *P. fluorescens* per mouse (George *et al.*, 2000). *P. fluorescens* was recovered in the intestinal tract after 3 hours but was completely cleared after the first day. *P. fluorescens* was detected in lungs, intestinal tract (small, large, cecum), MLN, spleen, and liver 3 hours after treatment but had completely cleared from all organs and tissues 2-d after treatment. At the completion of the study all mice treated with *P. fluorescens* appeared healthy and conventional indicators of mortality such as ruffled fur, lethargy, weight loss, conjunctivitis were not present.

As a result of these studies the U. S. EPA determined that the use of *P. fluorescens* as a microbial pest control for agricultural products (peas, snap bean, sweet corn) was "safe" and was exempt

from the requirement of a tolerance for a residue (USEPA, 1997). Their opinion was based on the fact that no harm should result from residues of *P. fluorescens* on treated vegetables because of the ubiquitous nature of this bacterium and its low toxicity to humans. EPA gave similar reasoning for granting an exemption from the requirement of a tolerance for residues of *P. fluorescens* in agricultural commodity mushroom. Again they cited that *P. fluorescens* is naturally occurring organisms and is the dominant microflora found in mushroom caps (USEPA, 1994).

Role of *Pseudomonas fluorescens* as an Opportunistic Pathogen

The human immune system provides a defense mechanism for rapid, highly specific, protective response against potentially pathogenic microorganisms. The immune system is mediated by complex multicellular and physiological mechanisms that allow an individual to distinguish foreign matter from self and eliminate or neutralize foreign material (Paul 1999). This system provides the mechanism to initiate rapid and highly specific responses to potentially pathogenic organisms. Loss of immune competence (*e.g.* immunosuppression) allows the opportunity for infection with organisms that are not usually pathogenic (Klassen 2001). Examples of compromised immune systems occur in patients undergoing cancer therapy where immunosuppressive drugs are often given or in individuals who have contracted AIDS (acquired immunodeficiency syndrome).

One clinical consequence of immunosuppression or immunodeficiency is an increased incidence of infectious disease. Often organisms that do not usually cause disease because they are well controlled by a normal immune response (opportunistic pathogens) may infect immunocompromised individuals. For example, *Pseudomonas fluorescens* has been implicated as opportunistic pathogen in immunocompromised individuals (Bloch *et al.*, 1981, Meguro *et al.*, 1983, Hessen *et al.*, 1987, Hsueh *et al.*, 1998, Prescott *et al.*, 1998, Rahiala *et al.*, 1998, Zervos *et al.*, 1998, Manfredi *et al.*, 1999). This microorganism has on occasion been isolated from patients with AIDS (Franzetti *et al.*, 1992; Roilides *et al.*, 1992), where it caused bacteremia and urinary tract, ocular and soft tissue infections. In a separate study Chamberland and coworkers (1992) conducted an across-Canada survey of septicemia where they found that 1.5% of isolates were *P. fluorescens*. The Organisation for Economic Co-operation and Development (OECD) also indicated that it is apparent that *P. fluorescens* can be an opportunistic pathogen in cancer patients and in other individuals who are severely immunocompromised (OECD, 1997).

Although *P. fluorescens* has been encountered in diseased or infected individuals the microorganism's pathogenic role in many of these cases is questionable or at best ill-defined. For example, Graevenitz, and Weinstein (1971) examined 43 clinical isolates of *P. fluorescens* from human sources. They concluded that clinical strain of *P. fluorescens* isolated from patients with compromised defenses did not play any etiologically defined role and pathogenic potential of these organisms was low in "normal" men. In an earlier report, Liu (1964) demonstrated that inability of *P. fluorescens* to produce infection in warm-blooded animals was due to its inability to grow at internal body temperatures. Because *P. fluorescens* is unable to grow at elevated temperatures like that of the human body (Palleroni, 1992), it is unlikely to be more than a rare opportunistic pathogen for warm-blooded animals (OECD 1997). Based on the results from these and other studies the virulence of *P. fluorescens* for normal (immunocompetent) human hosts is very low.

Contamination of Blood Products and Medical Devices

Pseudomonas species as a group are ubiquitous saprophytes with low virulence. They are primarily opportunistic pathogens to hosts with low resistance. *Pseudomonas* species are metabolically versatile, can grow on simple organic substrates, are prolific colonizers of surfaces, and can remain viable for long periods of time in a wide variety of habitats. *P. fluorescens* is unable to grow at elevated temperatures like that of the human body (Palleroni, 1992) and is unlikely to be more than a rare opportunistic pathogen for warm-blooded animals. However, this organism has the ability to grow at 4 °C (Gilardi, 1991). The ability of *Pseudomonas* species to grow under a wide range of nutritional conditions, to grow at low temperatures (less than 5 °C), and their wide distribution in the environment provide the potential and opportunity for the accidental contamination of whole blood, blood products, and medical devices (Puckett *et al.*, 1992; Stenhouse and Milner, 1992).

Bacterial contamination of the blood supply can occur via an endogenous or an exogenous source. Typically, Gram-negative "psychrophilic" species such as *P. fluorescens* survive storage at 4 °C. They may be cultured from refrigerated blood and can be implicated in transfusion reactions (Gibaud *et al.*, 1984., Murray *et al.*, 1987, Scott *et al.* 1988, Gottlieb and Anaesth, 1993). For example, Kabbaz and coworkers (1984) conducted laboratory studies demonstrating that small inocula of *P. fluorescens* proliferated in refrigerated fresh whole blood and reached 10^6 to 10^7 colony-forming units per milliliter seven days after incubation. They concluded that prolonged storage of blood was an important risk factor for the development of transfusion-related septicemia.

Pseudobacteremia may result from the infusion of contaminated products (Scott *et al.*, 1988; Simor *et al.*, 1985, Collignon *et al.*, 1999, Gottlieb *et al.*, 1991; Foreman *et al.*, 1991). For example, Smith and coworkers (2002) reported an outbreak of 38 cases of pseudobacteraemias caused by *P. fluorescens* in pediatric patients over a 10-month period. The source of the outbreak of pseudobacteraemia was found to be contaminated lithium heparin tubes, which were being filled prior to the filling of the blood culture bottle.

P. fluorescens has also been reported to cause infection from the use of contaminated equipment (Anderson and Davey, 1994). For example, Foulon *et al.* (1981) implicated *P. fluorescens* with an infection caused by a copper intrauterine device.

***In Vitro* Cytotoxic Effects of a *P. fluorescens'* Pigments**

Many *Pseudomonas* species produce an extracellular compound with yellowish green fluorescence, called pyoverdin, which functions as a siderophore. Siderophores are low-molecular-weight iron chelators that have very high affinity for iron. Pyoverdins act as extracellular solubilizing agents for iron from minerals or organic compounds. (Rombel and Lamont IL 1992). Fluorescent pigments are produced abundantly in media low in iron content and pyoverdin production is characteristic of most fluorescent species. At least 12 different pyoverdins have been identified among the various strains of *P. fluorescens*.(Meyer *et al.*, 1998, Mohn *et al.*, 1990). Pyoverdin production in *P. fluorescens* is dependent on the strain and the growth conditions (*e.g.*, iron availability) and Palleroni (1984) reported that *P. fluorescens* Biovars II and V, along with *P. chlororaphis* and *P. putida* Biovar B, have variable (11-89% positive) pyoverdin production.

Pyoverdins have been reported to adversely affect human leukocytes (Beccera *et al.* 2001). *In vitro* assays conducted with purified pyoverdin from selected strains of *P. fluorescens* strain have exhibited limited cytotoxic effects (Pajaro *et al.*, 1994, Pajaro *et al.*, 1995). Pajaro and Albesa (1990) examined a strain of *P. fluorescens* which grew and produced pigment in refrigerated human blood at 4 °C. This pigment, purified from *P. fluorescens* strains grown on synthetic medium, exhibited cytotoxic effects on human leukocytes. It is not clear if these antagonistic effects were directly related to the ability of the pyoverdin to remove essential components (*e.g.*, iron) from the tissue culture medium or if toxicity was due to some other mechanisms. In contrast to the cytotoxic effect described above, the pyoverdins have been shown to be effective in protecting human hepatocytes against the toxic effect of iron. Jego and coworkers (1997) reported that iron citrate was highly toxic for human hepatocytes. Addition of pyoverdin from *P. fluorescens* was able to ameliorate the toxic effects to the human hepatocytes by both decreasing the uptake of the iron by the cells and increasing its release from the loaded cells.

Search 3: Toxin Production by *Pseudomonas* species

The final literature survey was a limited search addressing toxin production by any member of the genus *Pseudomonas*. Over 629 references were retrieved and the results of this search demonstrated that toxin production occurs in a number of organisms from this genus. However, the majority of the toxins produced by *Pseudomonas* species have little to no clinical significance to humans and the toxicity is directed more towards plants. The one exception is Exotoxin A produced by *Pseudomonas aeruginosa* which is an important virulence factor in *Pseudomonas* disease in humans (Pollack 1983).

Many of the articles cited in this literature survey refer to strains of microorganisms that may no longer be classified as *Pseudomonas*. The genus *Pseudomonas* has recently undergone taxonomic reorganization and many of the strains originally described as species of the genus *Pseudomonas,* such as *Bulkhoderia cepacia,* have been reclassified into different genera (Kersters *et al.*, 1996). It is now generally recognized that the genus *Pseudomonas* (sensu stricto) should be limited to those organisms clustering with *P. aeruginosa* and *P. fluorescens* in the DNA-RNA homology group I, γ-subclass of the *Proteobacteria* (Moore *et al.*, 1996, Kersters *et al.*, 1996, Anzai *et al.*, 2000). This new definition brings the total number of *Pseudomonas* strains down to less than 30.

Conclusions

As noted, the literature searches retrieved approximately 800 references. The majority of these citations were not relevant to the search objectives and contained little information related to the toxigenicity or pathogenicity of *P. fluorescens*. However, a small subset of these citations was relevant as indicated and as discussed above.

P. fluorescens was shown not to be pathogenic in animal models. There were no reports of *P. fluorescens* being pathogenic in healthy humans, though there is some information on its role as an opportunistic pathogen in immune compromised hosts. The ability of *P. fluorescens* to grow at low temperatures (4 °C) has caused occasional problems in contamination of blood products or medical devices and there are sporadic reports of pseudobacteremia resulting from the infusion of contaminated products. However, these problems are likely not be specific to *P*

fluorescens, but rather are most likely related to the lipopolysaccharide (LPS; endotoxin) present in any gram-negative microorganism. *P fluorescens* also produces extracellular compounds, called pyoverdins, which function as a siderophores (iron binding proteins). *In vitro* assays conducted with purified pyoverdins from selected strains of *P. fluorescens* have shown limited cytotoxic effects to human leukocytes. It is not clear if these antagonistic effects were directly related to the ability of the pyoverdin to remove essential components (*e.g.*, iron) from the tissue culture medium or if toxicity was due to some other mechanisms.

Based on the results of this review the scientific literature was found to support the general view that *P. fluorescens* is considered to be nontoxigenic and nonpathogenic. Therefore, food or food ingredients safe for human consumption can be produced with this microorganism.

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15. Expert Panel Opinion

TO: David S. Hanselman, Ph.D.
Director of Regulatory Affairs, Environment, Health and Safety
Innovase LLC
5501 Oberlin Drive
San Diego, CA 92121

FROM: Michael W. Pariza, Ph.D. (Panel Chair)
University of Wisconsin
Madison, WI USA

Eric A. Johnson, Sc.D.
University of Wisconsin
Madison, WI USA

Ian C. Munro Ph.D., FRCPath
Cantox Health Sciences International
Mississauga, ON Canada

Robert A. Roth, Ph.D.
Michigan State University
East Lansing, MI USA

DATE: February 10, 2003

RE: **Expert Panel Opinion Regarding the Generally Recognized as Safe (GRAS) Status of Innovase's BD5088 α-amylase Preparation**

As independent experts qualified by relevant experience and scientific training to evaluate the safety of food additives, we, the undersigned Eric A. Johnson, Sc.D., Ian C. Munro, Ph.D., FRCPath, Michael W. Pariza, Ph.D., and Robert A. Roth, Ph.D., were asked by Innovase LLC (Innovase) to evaluate the safety of α-amylase BD5088, which is derived from *Pseudomonas fluorescens* Biovar I carrying an optimized α-amylase gene constructed from *Thermococcales sp.*, for use as a processing aid in the hydrolysis of edible starch to produce various starch hydrolysis products, including starch-derived sweeteners, and to produce fermentable sugars for use in the production of distilled ethanol for alcoholic beverages. *Curricula vitae* evidencing our qualifications for evaluating the safety of food additives are provided.

We independently and critically examined scientific information and data provided by Innovase, including information pertaining to the microbial genomics, source and structure of the BD5088 α-amylase gene, the safety of the recipient organism *Pseudomonas fluorescens* Biovar I, characterization of the BD5088 α-amylase expression product, the activity of BD5088 α-amylase enzyme, the method of manufacture and product specifications, analytical data, conditions of intended use, intended levels of use, estimated consumer exposure under the conditions of use, all unpublished corroborating toxicological and relevant safety studies conducted with this α-

amylase preparation provided by Innovase, and relevant information compiled from the published scientific literature.

Following independent, critical evaluation of the data and information, we convened by conference call in an Expert Panel meeting on December 20, 2002. At this meeting we concluded that Innovase's BD5088 α-amylase is manufactured in accordance with current Good Manufacturing Practices (cGMPs), meets the relevant food grade specifications as a processing aid in the hydrolysis of starch, and is "Generally Recognized As Safe" (GRAS) for food use under the conditions of use proposed by the manufacturer. A summary of the basis for our conclusion is provided below.

I. Manufacturing and Specifications

A comprehensive review of the relevant scientific literature indicates that species belonging to the genus *Thermococcales* are nonpathogenic and nontoxigenic for animals including humans. The derivation, source identity, and enzymatic activity of the optimized BD5088 α-amylase from *Thermococcales sp.* are fully described (Richardson *et al.*, 2002).[1] *Pseudomonas fluorescens* is nonpathogenic and nontoxigenic, and the BD5088 α- amylase gene recipient, *P. fluorescens* Biovar I strain MB101, was derived via safe strain lineage.

The BD5088 α-amylase gene was stably inserted into *P. fluorescens* Biovar I strain MB101. The entire genome of the recipient strain and both inserted plasmids have been sequenced to account for all introduced DNA. The production of BD5088 α-amylase follows multi-step fermentation of the recombinant *P. fluorescens* Biovar I, with induction of gene expression through the addition of high purity isopropyl β-D-thiogalactopyranoside (IPTG). BD5088 α-amylase is recovered and purified by standard industry processes.

Quality control measures ensure that viable *P. fluorescens* organisms do not survive the post-fermentation processing and are not present in the final α-amylase preparation. The absence of production organism is incorporated as a final product specification for BD5088 α-amylase. Additionally, microfiltration and, if necessary, treatment with high purity endonuclease or acid hydrolysis removes residual DNA, including antimicrobial resistance marker sequences, from the α-amylase preparation. All substrate materials and processing aids used in the production of Innovase's BD5088 α-amylase preparation are of high purity and of suitable food grade quality. The fermentation process is checked for culture purity and identity at regular intervals. The final BD5088 α-amylase preparation is formulated through the addition of approved food grade diluents, stabilizers and preservatives.

Specifications for the final α-amylase preparation meet acceptable food grade specifications and are consistent with Food Chemicals Codex[2] and Joint FAO/WHO Expert Committee on Food Additives[3] specifications for enzymes used in food processing. The Panel reviewed the results of analysis for nonconsecutive representative lots of BD5088 α-amylase preparation and concluded a demonstration of compliance with final product chemical, physical, and microbiological specifications.

II. Intended Use and Estimated Dietary Exposure

Innovase's BD5088 α-amylase is intended for use as a processing aid in the hydrolysis of edible starch to produce starch hydrolysis products, including starch-derived sweeteners such as glucose syrup, high fructose corn syrup, and dextrose, and to produce distilled ethanol for use in alcoholic beverages.

As a processing aid in the hydrolysis of starch, the intended level of use is 500 g α-amylase preparation per metric ton of starch. The Panel was presented with data demonstrating the glucose yield and carbohydrate profile of starch hydrolysis with BD5088 compared to other commercially approved α-amylase preparations. The Panel concluded that BD5088 α-amylase preparation is substantially equivalent to existing α-amylase preparations used in the food industry.

The Panel noted that IPTG was added during the manufacture of BD5088 α-amylase and was provided data on residual IPTG in final BD5088 α-amylase preparations and starch hydrolysis products. The Panel considered potential consumer exposure to IPTG to be insignificant (<0.5 ppb in food) and not of concern to safety under the conditions of intended use of BD5088 α-amylase.

The Panel concluded that consumer exposure to endonuclease would not be expected to occur given that the amount of high purity endonuclease added in the manufacturing process of BD5088 α-amylase preparation is very low (~1 ppb), and the enzyme is not stable under the pH and temperature conditions of intended use for BD5088 α-amylase.

For consumers of products prepared using BD5088 α-amylase, estimates of dietary exposure to this α-amylase presented to the Panel were extremely low. Residual BD5088 α-amylase could not be detected in glucose syrup to a limit of detection (LOD) of 2.4 ppb. Assuming residual BD5088 α-amylase to be present in all starch-derived sweeteners at 1.2 ppb (half of the LOD), an estimated intake of 3.6 μg total organic solids (TOS)/person/day in upper percentile consumers was calculated. This level of exposure is $>10^6$ times lower than the No Observed Adverse Effect Level (NOAEL) in animal toxicity studies.

III. Safety

The safety of BD5088 α-amylase was assessed using the decision tree strategy developed by Pariza and Johnson (2001)[4] for evaluating the safety of microbially derived food enzymes. The Panel recognized that all criteria of this strategy had been satisfied to conclude that α-amylase BD5088 is acceptable for use as a food enzyme.

The *Thermococcales sp.* source for the BD5088 α-amylase gene is non-toxigenic and non-pathogenic. The gene encoding this enzyme has been fully characterized and stably inserted into *P. fluorescens* Biovar I. *P. fluorescens* Biovar I strain MB101 is non-toxigenic and non-pathogenic and is the product of a safe strain lineage. Innovase's BD5088 α-amylase preparation contains no viable production organisms and no residual genetic material encoding for antibiotic resistance. The amino acid sequence of BD5088 α-amylase has been completely determined,

and its similarity and homology to other α-amylases, including those used in food production, has been demonstrated. The activity of BD5088 α-amylase is substantially equivalent to that of α-amylases from other microbial sources with history of safe use in food production.

The safety of BD5088 α-amylase and *P. fluorescens* was evaluated using appropriate methods and procedures. The Panel reviewed a battery of toxicological studies commissioned by Innovase. These studies included subacute and subchronic toxicity studies in rats, pathogenicity and toxigenicity studies in mice, and *in vitro* and bioinformatic studies related to evaluating allergenic potential. The results of these studies provided no evidence of potential adverse effects and support an overall conclusion of safety for this enzyme under the conditions of intended use.

In 14- and 90-day oral gavage studies with male and female Fischer 344 rats given doses of 71, 220, 710 or 2,200, and 89, 270 or 890 mg BD5088 α-amylase preparation (as TOS)/kg body weight/day, respectively, localized inflammation of the nasal and pulmonary mucosa was reported; however, the response was not dose-dependent, but was suggestive of aspiration of test material by the treated animals. Aspiration of the test substance is not uncommon in toxicology studies with doses administered by gavage. Expert review of the histopathology of the reported lesions concluded the response was consistent with a respiratory tissue inflammatory response to inhaled lipopolysaccharide (endotoxin), likely attributable to aspiration of the administered substance, either during or after gavage. To further investigate the response reported in the gavage studies, a 14-day dietary feeding study of rats was conducted with doses of 360 and 310 mg TOS/kg body weight/day for males and females, respectively. No inflammation in the respiratory tract, or any other adverse effect, was observed with these high doses of BD5088 α-amylase in the diet, thereby supporting the interpretation that airway inflammation observed in the gavage studies was due to aspiration of the test substance. Endotoxins are essentially nontoxic via the oral route and are not considered to be a safety issue for food processing enzymes. Additionally, the Panel concluded that exposure to endotoxin would be negligible and of no toxicological concern given the very low estimated intake of BD5088 α-amylase.

Evidence available in the scientific literature indicates that *P. fluorescens* is neither pathogenic nor toxigenic for humans. To evaluate the pathogenic and toxigenic potential of *P. fluorescens* Biovar I strain MB101 used to produce BD5088 α-amylase, Balb/C mice were administered a single dose concentrate of microorganisms (up to 6×10^8 CFU) or filtered spent culture medium, by gavage, and evaluated over a 21-day period. The results of this study demonstrated no evidence of infection or colonization in mice administered high numbers of viable *P. fluorescens* Biovar I strain MB101 indicating an absence of pathogenicity. Independent expert review of this study by Dr. Stanley Falkow (Stanford University School of Medicine) concluded *P. fluorescens* Biovar I strain MB101 to be non-pathogenic. Mice administered spent culture medium demonstrated no adverse effects indicating no evidence of toxigenicity of *P. fluorescens* Biovar I strain MB101.

As with many other inhaled proteins, occupational allergies to α-amylases have been reported in the literature; however, sensitization and allergic reactions following consumption are not common. The allergenic potential of BD5088 α-amylase was evaluated using bioinformatic methods and *in vitro* digestibility studies consistent with Food and Agriculture Organization of

the United Nations/World Health Organization (FAO/WHO)[5] recommendations pertaining to novel proteins in the diet. Protein sequence comparisons using comprehensive databases of known allergen sequences demonstrated BD5088 α-amylase has no critical sequence homology to known allergens (*i.e.*, no matches of at least 8 contiguous amino acids) and no structural similarity (*i.e.*, <35% shared amino acid identity with known allergens over segments of at least 80 amino acids). BD5088 α-amylase is not stable under simulated conditions of digestion with pepsin. These results, in combination with the very low potential dietary exposure to BD5088 α-amylase, provide no suggestion of potential allergenicity as a safety concern for Innovase's α-amylase BD5088.

In its entirety, the scientific evidence reviewed by the Panel supports the intended use of Innovase's α-amylase BD5088 as a processing aid in the production of food grade starch hydrolysis products and its designation as GRAS.

Conclusion

Based on our independent and collective critical evaluation of the available pertinent scientific evidence, we conclude that Innovase's BD5088 α-amylase, which is derived from *Pseudomonas fluorescens* Biovar I carrying a optimized α-amylase gene constructed from *Thermococcales sp.*, is manufactured in accordance with cGMPs, meets the relevant food grade specifications and, based on scientific procedures, is "Generally Recognized as Safe" (GRAS) for use in food as specified herein.

Eric A. Johnson, Sc.D.
University of Wisconsin
Madison, WI USA

(b)(6)

Date: Feb 17, 2003

Ian C. Munro, Ph.D., FRCPath
Cantox Health Sciences International
Mississauga, ON CAN

(b)(6)

Date: Feb 12/03

Michael W. Pariza, Ph.D. *(Panel Chair)*
University of Wisconsin
Madison, WI USA

(b)(6)

Date: Feb. 17, 2003

Robert A. Roth, Ph.D.
Michigan State University
East Lansing, MI USA

(b)(6)

Date: Feb 21, 2003

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16. CV: Johnson

Appendix 16

CURRICULUM VITAE

Dr. Eric A. Johnson

Education

University of California-Davis	B.S.	1976	Fermentation Science
University of California-Davis	M.S.	1978	Food Science
Massachusetts Institute of Technology	Sc.D.	1983	Food Microbiology
Harvard Medical School	Postdoc	1985	Bacterial Physiology and Genetics

Positions

1974 to 1976	Undergraduate Research Fellow, University of California-Davis
1976 to 1978	Graduate student for the M.S. degree, University of California-Davis
1978 to 1979	Staff Research Associate, Aquaculture Program, University of California-Berkeley
1980 to 1983	Graduate student for Sc.D. degree, Massachusetts Institute of Technology
1983 to 1985	National Institutes of Health Postdoctoral Research Fellow
1984	Assistant Instructor of Microbiology and Molecular Genetics, Harvard Medical School
1985 to 1990	Assistant Professor of Bacteriology, University of Wisconsin-Madison
1985 to 1990	Assistant Professor of Food Microbiology and Toxicology, University of Wisconsin-Madison
1991 to Present	Affiliate Faculty of the Center for Dairy Research, University of Wisconsin-Madison
1990 to Present	Associate Professor of Bacteriology, University of Wisconsin-Madison
1990 to 1996	Associate Professor of Food Microbiology and Toxicology, University of Wisconsin-Madison
1996 to Present	Professor of Food Microbiology and Toxicology, University of Wisconsin Madison

Awards and Other Professional Activities

1975 to 1976	President's Undergraduate Fellowship, University of California-Davis
1976	Western United States Undergraduate Research Award, Institute of Food Technologists
1976	Jastro-Shields Scholarship, University of California-Davis
1977	Sigma XI Grant-in-Aid of Research
1977 to 1978	SeaGrant Graduate Fellowship
1980 to 1981	Department of Energy Graduate Fellowship
1981 to 1983	National Distiller's Graduate Fellowship
1982 to 1983	National Science Foundation Research Grant, 1982 - 1983.
1996	Chair Elect, Division P (Food Microbiology), American Society for Microbiology
1999	Educator Award, International Association for Food Protection
2000	Elected to Fellowship in the American Academy of Microbiology
2001 to 2005	Editor for Applied and Environmental Microbiology
2001	Appointed to AIBS panel to review DOD Toxicology Program, Fort Detrick, Maryland

2001	Involved in CDC program for bioterrorism preparedness
2001-2002	NIH study panel for bioterrorism related research proposals

Journal Publications

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Johnson, E.A. and B.F. Brehm-Stecher. Method of sensitizing microbial cells to antimicrobial compound. U.S. Patent #6,319,958, November 20, 2001.

Johnson, E.A., M.C. Goodnough, M. Bradshaw, and W.H. Tepp. Hybrid botulinal neurotoxins. U.S. Patent #6,444,209, September 3, 2002.

Appendix 17

2233 Argentia Road, Suite 308
Mississauga, ON L5N 2X7
Phone: 905-542-2900
Fax: 905-542-1011

CURRICULUM VITAE
IAN CRAIG MUNRO

Telephone: (905) 542-2900 E-mail: imunro@cantox.com Fax: (905) 542-1011

EDUCATION

1970	Ph.D., Toxicology and Pharmacology, Queen's University, Kingston, Ontario, Canada
1967	M.Sc., Nutrition, McGill University, Montreal, Canada
1962	B.Sc., McGill University, Montreal, Canada

ACCREDITATION

1999	Fellow of The Academy of Toxicological Sciences
1988	Fellow of Royal College of Pathologists, London, England

EMPLOYMENT HISTORY

1999-Present	**University of Toronto,** Department of Nutritional Sciences, Faculty of Medicine. Toronto, Ontario. Professor.
1999-Present	**CANTOX Health Sciences International,** Mississauga, Ontario. President.
1985-1999	**CanTox Inc.,** Consultants in Health and Environmental Sciences. Mississauga, Ontario, Consultant Toxicologist & Principal.
1983-1992	**Canadian Centre for Toxicology,** Guelph, Ontario, Canada. Director.
1981-1983	**Health and Welfare, Canada**, Food Directorate, Health Protection Branch. Ottawa, Canada. Director General.
1976-1981	**Health and Welfare, Canada**, Bureau of Chemical Safety, Food Directorate, Health Protection Branch, Ottawa, Canada. Director.
1975-1976	**Health and Welfare, Canada**, Bureau of Chemical Safety, Health Protection Branch, Ottawa, Canada. Chief, The Division of Toxicology.
1974-1976	**Health and Welfare, Canada**, Bureau of Chemical Safety, Health Protection Branch. Ottawa, Canada. Section Head, The Division of Toxicology.
1963-1974	**Health and Welfare, Canada**, Health Protection Branch, Ottawa, Canada. Research Scientist.

COMMITTEE MEMBERSHIPS

2002-	Technical Advisory Committee, World Food Program (WFP), The Food Aid Organization of the United Nations
2001	Chairman, Safety Assessment of Foods Derived from Genetically Modified Microorganisms. World Health Organization, Headquarters, Geneva, Switzerland – September 2001
2000-Present	Member, Georgetown Dialogue Science Council, Georgetown University Center for Food and Nutrition Policy (CFNP)

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2000-Present	Consultant, FEMA Expert Panel
1999	Center for Food Safety and Applied Nutrition (CFSAN) Research Program Committee, Food and Drug Administration
1998-2001	Member, Minister's Advisory Board, Canadian Food Inspection Agency
1996-2002	Chairman, Institute of Medicine, Subcommittee on Upper Safe Reference Levels of Nutrients
1996	Member, Ad Hoc Expert Panel, Life Sciences Research Office, Federation of American Societies for Experimental Biology (FASEB)
1993	Member FAO/WHO Expert Committee on Food Additives
1989	Chairman, Expert Group to Develop a Threshold of Regulation for Indirect Food Additives
1989-1991	Member, Scientific Committee, International Food Biotechnology Council
1985-2000	Member, FEMA Expert Panel
1985	Member ILSI-NF, Nutrition and Safety Committee (FNSC)
1985	Member, NAS, Committee on Carcinogenicity of Cyclamates.
1984	Member, Committee on Food Chemicals Codex.
1983-1984	Member, Panel of Chemical Carcinogenesis Testing and Evaluation (National Toxicology Program)
1983	Member, The Nutrition Foundation Project on the Use of Mouse Hepatoma Data.
1981-1983	Expert Committee on the Relevance of Mouse Liver as a Model for Assessing Carcinogenic Risk, The Nutrition Foundation, Inc.
1981-1982	Expert Advisory Committee to The Nutrition Foundation, Inc., on the Assessment of the Safety of Lead and Lead Salts in Foods.
1981	Chairman, International Committee on Hazards Associated with Dioxin in the Great Lakes.
1981	Chairman, WHO Ad Hoc Meeting on the Future of Joint Expert Committees in the Context of the International Program on Chemical Safety, Geneva.
1980-1983	Chairman, Health Protection Branch/Food Industry Liaison Committee.
1980-1983	Chairman, Interdepartmental Committee on Canning Regulations.
1980	Member, Federal Interdepartmental Salmonella Committee.
1980	Member, Senior Level Committee (U.S., U.K., Canada).
1980	Member, International Life Sciences Institute Experts in Pathology and Toxicology.
1980	Member, Technical Committee: WHO International Program on Chemical Safety.
1978-1980	Expert Committee on Food Safety - Agriculture Canada
1978-1980	Food Safety Council, Social and Economic Committee.
1978-1979	U.S. National Academy of Sciences, Subcommittee on Risk Assessment - Safe Drinking Water Committee.
1978	Chairman, Tripartite Toxicology Committee (U.S., U.K., Canada).
1977-1981	International Commission for Protection Against Environmental Mutagens and Carcinogens (ICPEMC), subcommittee 3.
1977-1979	U.S. National Cancer Institute, Cause and Prevention Scientific Review Committee.
1976-1984	WHO/FAO Joint Expert Committee on Food Additives.
1976-1980	Food Safety Council, Toxicology Committee.
1976-1979	Canadian Council on Animal Care.
1976-1979	Interdepartmental Committee on Toxicology Needs in Canada.
1976-1978	National Research Council Task Force on Mercury and Captan.
1975-1976	U.S. National Academy of Sciences Committee on Toxicology
1975-1976	WHO/FAO Committee on Criterion Documents on the Toxicology of Environmental Chemicals.

EDITORIAL RESPONSIBILITIES

1982-1996	Editorial Board	Journal of the American College of Toxicology
1979-1991	Advisory Board	Neurotoxicology
1978-1989	Editorial Board	Journal of Environmental Pathology and Toxicology

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PROFESSIONAL AFFILIATIONS

Professional Society Memberships:

Member, Society of Toxicology
Member, Toxicology Forum
Member, Society of Toxicology of Canada
Member, American College of Toxicology
Member, Institute for Risk Research
Member, International Society of Regulatory Toxicology and Pharmacology

Contributions to Professional Societies:

1981	Professional Standards Evaluation Board in General Toxicology, Academy of Toxicological Sciences
1978-1979	Society of Toxicology, Nominating Committee
1978-1979	Society of Toxicology, Finance Committee
1976-Present	Toxicology Forum, Inc., Board of Directors

AWARDS

1998	International Society of Regulatory Toxicology and Pharmacology "International Achievement Award" for his guiding role as Chairman of the Expert Panel of Members – "Interpretive Review of the Effects of Chlorinated Organic Chemicals".
1975	Society of Toxicology "Achievement Award" for outstanding contributions to the science of toxicology by an individual 35 years of age or younger.

SCIENTIFIC PUBLICATIONS AND MONOGRAPHS

Munro, I.C., Harwood, M, Hlywka, J.J., Stephen, A.M., Doull, J., Flamm, W.G., and Adlercreutz, H. 2002. Soy Isoflavones: A Safety Review. Nutr Rev. In press.

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Kroes, R., Galli, C., Munro, I., Schilter, B., Tran, L.-A., Walker, R., and Würtzen. 2000. Threshold of Toxicological Concern for Chemical Substances Present in the Diet: A Practical Tool for Assessing the Need for Toxicity Testing. Reprinted from Food Technol 38(2-3):255-312.

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PRESENTATIONS

Munro, I.C., and Roberts, A.S. 2002. Functional Foods and Nutraceuticals -- How to Launch Nutraceuticals on the U.S. Market. A workshop conducted by Dr. Ian Munro and Dr. Ashley Roberts in association with Archimex, November 26, Paris, France.

Munro, I.C. 2002. Setting Tolerable Upper Intake Levels for Nutrients. Presented at the workshop on "Dietary Reference Intakes and Discretionary Fortification". Sponsored by the Committee on Use of Dietary Reference Intakes in Nutrition Labeling of the Food and Nutrition Board, Institute of Medicine, November 21, Washington, DC.

Munro, I.C. 2002. Setting Tolerable Upper Intake Levels for Nutrients. Presented at the American Dietetic Association, Food & Nutrition Conference & Exhibition 2002, October 21, Philadelphia, PA.

Munro, I.C. 2002. Regulatory and Safety Requirements for Obtaining GRAS Status. Presented at the American College of Nutrition's 43rd Annual Meeting, October 3, San Antonio, TX.

Munro, I.C. 2002. The JECFA Procedure for the Safety Evaluation of Flavoring Substances. Presented at the JECFA Symposium organized by the Japanese Flavor & Fragrance Material's Association (JFFMA), September 26, Tokyo, Japan.

Munro, I.C. 2002. Risks From Acrylamide in Food. Presented at the Ceres Roundtable: Acrylamide: Lessons Learned, Plans Ahead, September 9, Virginia Tech, Alexandria, VA.

Munro, I.C. 2002. The Precautionary Principle and the Scientific Risk Assessment Process. Presented at the International Society of Regulatory Toxicology and Pharmacology Meeting, June 21-22, Arlington, VA.

Munro, I.C. 2002. OECD/FAO Substantial Equivalence Framework for Whole Food Safety Assessment. Presented at the 41st Annual Meeting & ToxExpo, March 17-21, Nashville, TN.

Munro, I.C. 2001. Dietary Exposure from Migration of Packaging Materials. Presentation at the Joint JRC/ILSI Europe Workshop on Exposure from Food Contact Materials, October 15-16, Ispra, Italy.

Munro, I.C. 2001. Safety Evaluation of Foods Derived from Genetically Modified Crops. Presented at the 222nd American Chemical Society Meeting, August 29, Chicago, IL.

Munro, I.C. 2001. Appropriate Use of Preclinical Data in Drug Development. Presented at the joint meeting of the Michigan Chapter of the Society of Toxicology (MISOT) and the Michigan Society for Medical Research (MISMR), May 18, Ann Arbor, Michigan.

Munro, I.C. 2001. Risk Analysis of Food Derived from Genetically Modified Plants. Presented at the Food and Agriculture Organization of the United Nations' (FAO) "Seminar on Risk Analysis for Food Control: A Practical Approach Through Case Studies" organized jointly with ILSI and the University of Brasilia, May 9-11, Brasilia, Brazil.

Munro, I.C. 2000. Safety Evaluation of Foods Derived from Genetically Modified Crops. Presented at the Brazilian Association of Food Industries' "Safety Assessment of Biotechnology Derived Foods" seminar, December 5, 6 & 7, São Paulo, Brazil.

Munro, I.C. 2000. Risk Assessment of Packaging Materials. Presented at the 2nd International Symposium on Food Packaging. Ensuring Safety and Quality of Foods, November 8-10, Vienna, Austria.

Munro, I.C. 2000. EUROTOX/SOT Debate. An evaluation demonstrating that foods derived from GM crops are as safe as their traditional counterparts is an appropriate paradigm for assessing the safety of genetically modified foods. @ For the motion: Ian C. Munro (SOT). EUROTOX 2000, XXXVIII European Congress of Toxicology, September 17-20, London, England.

Munro, I.C. 2000. Safety of Foods Produced by rDNA Technology. Presented at the Institute of Medicine/Food and Nutrition Board Meeting, July 20, Woods Hole, MA.

Munro, I.C. 2000. Society of Toxicology/EUROTOX Debate Presentation. 2000 Society of Toxicology Annual Meeting, March 21, Philadelphia, PA.

Munro, I.C. 2000. Developing Integrated Scientific & Regulatory Strategies, Resolving Complex Scientific Issues, and Facilitating Timely Regulatory Approvals. TNO Nutrition and Food Research Institute, February 29, Zeist, The Netherlands.

Munro, I.C. 2000. Applying a Threshold of Regulation Concept to the Safety Evaluation of Packaging Materials. Nutripack Food & Beverage Packaging Congress, January 26-27, Paris, France.

Munro, I.C. 1999. Key Safety Issues in Bringing a Functional Food or Nutraceutical to Market. Nutraceutical Opportunities Summit, December 8-9, Toronto, Ontario.

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Munro, I.C., Bechtel, D., Schinkel, H., and McColl, D. 1999. Functional Foods: International Comparisons of the Scientific and Regulatory Attributes Affecting Product Development and Market Access.

Munro, I.C., McColl, D., Bailey, R., Coutrelis, N., and Schinkel, H. 1999. Special Forum: International Regulatory Issues in Marketing Functional Foods: Barriers and Opportunities. Institute of Food Technologist's Annual Meeting, July 24-28, Chicago, IL.

Munro, I.C. 1999. 1) Safety Assessment of Process Flavors. 2) Perspective of the Food and Nutrition Board's Subcommittee on Upper Reference Levels of Nutrients. 1999 Annual Summer Meeting of The Toxicology Forum, July 12 - 16, Aspen, Colorado.

Munro, I.C. 1999. The Crucial Role of Safety and Efficacy Principles for Nutraceuticals, Functional and Medical Foods - Nutraceutical, Functional & Medical Foods Conference, May 6-7, Toronto, Ontario.

Munro, I.C. 1999. Assessing the Safety of Flavoring Substances. Flavor and Extract Manufacturers' Association of the United States - 90th Annual Convention, May 2-5, Palm Beach, Florida.

Munro, I.C. 1999. Concepts in Safety Evaluation of HPV Food Substances. Vision 20/20 Workshop - TestSmart - A Humane and Efficient Approach to SIDS Data, April 26-27, Fairfax, Virginia.

Munro, I.C. 1999. Effect of Intake Level on the Safety Evaluation of Flavoring Substances. Scientific Committee on Food - DGIII - DGXXIV Joint Workshop on Chemically Defined Flavouring Substances, March 25, Brussels, Belgium.

Munro, I.C., Berndt, W., Borzelleca, J., Flamm, G., Lynch, B., Kennepohl, E., Bär, A., and Modderman, J. 1999. Erythritol: An Interpretive Summary of the Biochemical, Metabolic, Toxicological and Clinical Data. Poster presentation at the Society of Toxicology Annual Meeting, March 14-18, New Orleans, Louisiana.

Munro, I.C. 1998. 1) A Global Perspective on Regulatory Approval for Food Ingredients, Nutraceuticals, and Dietary Supplements. 2) Gaining Product Approval in Canada. 3) Key Elements in Formulating a Global Regulatory Plan. International Food Technologist's 1998 Pre-Annual Meeting Continuing Education Program #5, June 19 & 20, Atlanta, GA.

Munro, I.C. 1998. FNB Model for Development of Tolerable Upper Intake Levels. Presented at the European Toxicology Forum Meeting, May 13, Brussels, Belgium.

Munro, I.C. 1998. International Perspectives for Ensuring Safe Food. Presented at the Institute of Medicine, April 29, Washington, D.C.

Munro, I.C. 1997. The Development of Tolerable Upper Intake Levels for Nutrients. Presented at the Insight Information Inc. Conference - New Nutrition Recommendations - Capitalizing on New Opportunities, December 11, Toronto, Ontario.

Munro, I.C. 1997. A Model for the Development of Upper Levels. Presented at the Dietary Reference Intakes Conference - New Vision, New Challenges, Ontario Institute for Studies in Education, November 24, Toronto, Ontario.

Munro, I.C. 1997. A Model for the Development of Tolerable Upper Intake Levels for Nutrients. Presented at the Calcium Workshop, Program in Food Safety, University of Toronto, October 30, Toronto, Ontario.

Munro, I.C. 1997. A Model for the Development of Tolerable Upper Intake Levels for Nutrients. Presented at Dietary Reference Intakes: Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride Workshop, Institute of Medicine, September 23, Washington, D.C.

Munro, I.C., Daniels, J.M., and Lynch, B.S. 1997. A Review of the Safety of Vitamin B6 (Pyridoxine): Implications for Determining the Safe Upper Intake from Dietary Supplements. Presented at Vitamin B6: New Data, New Perspectives, Council for Responsible Nutrition, September 8, London, England.

Munro, I.C., and Kroes, R. 1997. Application of a Threshold of Regulation Concept in the Safety Evaluation of Certain Flavoring Substances. Presented at the Forty-ninth Meeting of the Joint FAO\WHO Expert Committee on Food Additives, June 17-26, Rome, Italy.

Bechtel, D., Locke, L., and Munro, I.C. 1997. Need for Scientific Substantiation for Functionality of Food Components for Health Promotion. Presented at the ILSI N.A. Workshop - The Future of Functional Foods for Health Promotion: A Public Health Opportunity?, June 4-5, Washington, D.C.

Munro, I.C. 1997. 2,4-D - Safety and Exposure. Presented to Poisons Centre staff, academic pharmacology staff and postgraduate students at the University of Dunedin, March 13, Dunedin, New Zealand.

Munro, I.C. 1997. 2,4-D - Safety and Exposure. Presented to toxicologists and occupational health specialists from the New Zealand Ministry of Agriculture and Ministry of Environment, March 12, Wellington, New Zealand.

Munro, I.C. 1997. Development of a Procedure for the Safety Evaluation of Flavouring Substances. Presented at the International Symposium on Flavours and Sensory Related Aspects, March 6-7, Cernobbio (Como), Italy.

Munro, I.C. 1996. 1) Current Issues in the Evaluation of the Safety of Food and Food Ingredients. 2) Issues in the Safety Assessment of Carbohydrate/Fat Substitutes. Presented at the ASCEPT Toxicology Workshop, June 17-18, Canberra, Australia.

Munro, I.C. 1995. Interpretive Review of the Potential Adverse Effects of Chlorinated Organic Chemicals on Human Health and the Environment. Report of an Expert Panel. Presented at Dioxin >95, 15th International Symposium on Chlorinated Dioxins and Related Compounds, August 21-25, Edmonton, Alberta.

Munro, I.C. 1995. The Safety Evaluation of Flavoring Substances: The GRAS Process. Presented at the Second Workshop - Harmonization and Food Safety, April 20-21, Hong Kong.

Munro, I.C., McGirr, L.G., Nestmann, E.R., and Kille, J.W. 1994. Macronutrient Substitutes: Safety Factor Alternatives And Human Mimetic Models. Thirty-third Annual Meeting of Society of Toxicology, Dallas, Texas.

Munro, I.C., McGirr, L.G., Nestmann, E.R., and Kille, J.W. 1994. Macronutrient Substitutes: Alternatives To Traditional Safety Testing. Annual Meeting of Institute of Food Technologists, Atlanta, Georgia.

Munro, I.C. 1993. Harmonization of Conventional Toxicology Studies - A Commentary. Presented at ILSI Conference on RedBook II, December 16, Washington, DC.

Munro, I.C. 1993. The Exposure and Toxicity of 2,4-D. Presented at The Toxicology Forum, Aspen, CO. (July).

Munro, I.C. 1992. Novel Foods, Workshop on Novel Foods and Novel Food Processes. Presented at the Program in Food Safety, Nutrition and Regulatory Affairs, Department of Nutritional Sciences, Faculty of Medicine, University of Toronto. Toronto, Ontario. (November).

Munro, I.C. 1992. Toxicology and Drug Development: Managing the Issues. Presented to Ciba-Geigy Canada Ltd., Mississauga, Ontario. (October).

Munro, I.C. 1992. Adverse Effects and Indoor Air Pollution. Presented at the Thirteenth Annual Meeting of the American College of Toxicology, San Francisco, CA. (October).

Munro, I.C. 1992. Toxicology of Benzoyl Peroxide. Presented at The Toxicology Forum, Aspen, CO. (July).

Munro, I.C., Borzelleca, J.F., and Squire, R.A. 1991. The Safety of Xylitol for Use in Food. Report of an Expert Panel.

Munro, I.C. 1991. Food Safety. Presented at a Food Safety Seminar Embassy of the United States, Ottawa, Ontario, Canada.

Munro, I.C., and Orr, J. 1991. Dioxins in Paper Products. Canadian Paediatric Society Workshop on Infant Diapers.

Munro, I.C., and Orr, J. 1991. The Saccharin Lesson. Presented at the Symposium on Chemical Carcinogenesis: The Relevance of Mechanistic Understanding in Toxicological Evaluation. Berlin, Germany.

Munro, I.C. 1991. Impact of Agricultural Activities on Health Risks From Drinking Water. Presented at the Interdisciplinary Symposium on Agriculture and Water Quality Centre for Soil and Water Conservation. University of Guelph, Ontario.

Munro, I.C. 1990. Scientific Aspects of the IFBC Report. Presented to the Toxicology Forum. Washington, DC.

Munro, I.C., and Hall, R.L. 1990. Food Safety and Quality - Impact of Biotechnology. Presented at the Agricultural Biotechnology, Food, Safety and Nutritional Quality for the Consumer Second Annual Meeting. Ithaca, New York.

Munro, I.C. 1990. Food Safety and Environmental Issues in the year 2010. Presented to the Western Canadian Wheat Growers' Association, Regina, Saskatchewan, Canada.

Munro, I.C. 1990 & 1989. Issues in Food Safety, "Later in Life Learning Series", Toronto Ontario and The Environmental Forum, Belleville, Ontario.

Munro, I.C. 1989. Issues to be Considered in the Safety Evaluation of Fat Substitutes. Presented at the Workshop on Re-evaluation of Toxicity Methodology including Gross Nutrients. Limelette, Belgium.

Munro, I.C. 1989. Natural Versus Man-Made. Presented to the Ontario Institute of Agrologists, Toronto, Ontario.

Munro, I.C. Neoplasm Promotion. Prepared for Environmental Health and Safety Council of The American Health Foundation.

Munro, I.C. 1986. Governmental Approach to Regulatory Priorities and Risk Management of Flavors and Fragrances. Presented at The Tenth International Congress of Essential Oils, Fragrances and Flavors. Washington, DC.

Munro, I.C. 1985. The Role of Toxicology in Strategies for Cancer Prevention. Presented at the American Society of Preventive oncology. Eighth Annual Meeting, Toronto, Canada.

Munro, I.C. 1985. The Ingredients of Foods: How They are Tested and Why They are Selected. Presented at the ILSI Workshop on Adverse Reactions to Foods and Food Additives, Orlando, Florida.

Munro, I.C., Goldberg, L., and Farber, E. 1985. Formaldehyde Risk Assessment. Report to Ontario Ministry of Labour.

Munro, I.C. 1984. Risk Assessment and Environmental Regulation. Prepared for the ILSI Symposium on Safety Assessment. Tokyo, Japan.

Munro, I.C. 1984. Report to the Royal Commission to Inquire into the Use and Effects of Chemical Agents on Australian Personnel in Vietnam.

Munro, I.C. 1983. Artificial Sweeteners (Saccharin) - General Review of Carcinogenicity Data. Presented at the Third European Toxicology Forum. Geneva, Switzerland.

Clayson, D.B., and Munro, I.C. 1983. Safety Evaluation of Low Levels of Toxic Agents in Food with Emphasis on Carcinogenesis and Mutagenesis. Presented at the International Symposium on the Safety Evaluation of Animal Drug Residues. Berlin, Germany.

Munro, I.C., and Bradshaw, L.R.A. 1983. Government Decision-Making with Incomplete Epidemiologic Evidence. Presented at the Canadian Society for Clinical Investigations Symposium in Clinical Epidemiology. Calgary, Alberta.

Munro, I.C. 1983. Overview of Factors that Influence Food Safety Decisions. Presented at the International Life Sciences Institute Symposium, Safety Assessment: Interface Between Science, Law and Regulation. Washington, DC.

1983. The Relevance of Mouse Liver Hepatoma to Human Carcinogenic Risk. Report of a Panel to the International Expert Advisory Committee to the Nutrition Foundation.

Munro, I.C. 1983. Introductory Remarks. Presented at the Toxicology Forum Meeting. Arlington, Virginia.

Charbonneau, S.M., and Munro, I.C. 1982. Dietary Factors Affecting Pesticide and Xenobiotic Toxicity. Presented as a Poster at the Fifth International Congress on Pesticide Chemistry. Kyoto, Japan.

Tryphonas, H., and Munro, I.C. 1982. Risk-Benefit Assessment in Immunotoxicology. Presented by Mrs. Tryphonas at NATO Advanced Study Institute on Immunotoxicology. Acadia University, Wolfville, Nova Scotia.

Munro, I.C., Miller, C.T., and Krewski, D. 1982. Regulatory Control of Environmental Chemicals: A Canadian Viewpoint. Presented at the First World Congress on Toxicology and Environmental Health. Washington, DC.

Munro, I.C. 1982. The Necessity for Compatible Standards. Presented at the 1982 Annual Winter Toxicology Forum Meeting, February 15-17, Arlington, Virginia.

Munro, I.C. 1981. Regulatory Concerns - Overview. Presented at the Fourteenth Annual Symposium of the Society of Toxicology of Canada. Montreal, Quebec.

Munro, I.C., and Krewski. 1981. Risk Assessment and Regulatory Decision Making. Presented at the Toxicology Forum Meeting, August 9-13, Vancouver, British Columbia.

Munro, I.C. 1981. Risk Assessment and Regulatory Decision Making. Presented at the 64th Chemical Conference and Exhibition, Chemical Institute of Canada, May 31-June 3, Halifax, Nova Scotia.

Munro, I.C. 1981. Science and Issues of Food Additive Use. Presented at Food Additives Symposium. University of Toronto, Faculty of Medicine and Program in Human Nutrition.

Munro, I.C., and Krewski, D.R. 1980. The Role of Risk Assessment in Regulatory Decision Making. Presented at the Thirteenth Annual Symposium of the Society of Toxicology of Canada, December 2-3, Montreal, Quebec.

Munro, I.C., and Krewski, D. 1980. The Role of Risk Assessment in Regulatory Decision Making. Presented at Symposium on Health Risk Analysis, October 27-30, Gatlinburg, Tennessee.

Munro, I.C. 1980. Scientific Evaluation of Benefit Risk Assessments in Food Safety. Presented at the Gordon Research Conference on the Microbiological Safety of Foods, June 16-20, Plymouth, New Hampshire.

Munro, I.C. 1980. Regulatory Control of Carcinogens. Presented at the Toxicology Forum Meeting, February 28-March 1, Arlington, Virginia.

Munro, I.C. 1979. Reproductive Toxicity. Presented at the International Course on the Principles and Methods in Modern Toxicology, October 22-24, Belgirate, Italy.

Munro, I.C. 1979. Scientific Evaluation of Benefit/Risk Assessments in Food Safety. Presented at the 29th Annual Meeting of the Institute of Food Technologists, June 10-13, St. Louis, Missouri.

Munro, I.C. 1978. Compilation of United States and Canadian Legislation Pertaining to Environmental Safety. Prepared for the International Commission for Protection Against Environmental Mutagens and Carcinogens.

Munro, I.C. 1978. Chapter on ADI Concept. Prepared for the Safe Drinking Water Committee, National Academy of Sciences.

Munro, I.C. 1978. Detecting and Measuring Carcinogens. Presented at the Law and Public Affairs Seminar on Government Regulation of Cancer-Causing Chemicals, December, Washington, DC.

Munro, I.C. 1978. Environmental Contaminants and Food Safety. Presented at the XI International Congress of Nutrition Conference, September, Rio de Janiero, Brazil.

Munro, I.C. 1978. Reproductive Toxicity and the Problems of *In Utero* Exposure. Presented at the International Symposium on Chemical Toxicology of Food, June, Milan, Italy.

Munro, I.C. 1978. Environmental Contaminants. Presented at the Symposium on Principal Hazards in Food Safety and Their Assessment, FASEB Annual Meeting, April, Atlantic City, New Jersey.

Munro, I.C. 1977. Regulatory Applications of Short-Term Tests for Carcinogenicity. Presented at the Gordon Research Conference, August, Meriden, New Hampshire.

Munro, I.C. 1977. Overview - Dose Selection. Presented at the Toxicology Forum Meeting, July, Aspen, Colorado.

Munro, I.C. 1977. The Importance of Specifications for Substances in Their Safety Evaluation in Foods. Prepared for the Scientific Committee of the Food Safety Council.

Munro, I.C. 1977. Working Papers for 34 Food Colors. Prepared for Joint FAO/WHO Expert Committee, Geneva.

Charbonneau, S.M., Munro, I.C., and Nera, E. 1977. Chronic Toxicity of Methylmercury in the Adult Cat. Proc. X Symposium on Trace Substances in Environmental Health, Columbia, Missouri.

Munro, I.C. 1976. Considerations in Chronic Toxicity Testing: The Chemical, The Dose, The Design. Presented at the Status of Predictive Tools in Application to Safety Evaluation Conference, November, Little Rock, Arkansas.

Munro, I.C. 1975. Working Paper on Nitrates, Nitrites and Nitrosamines. Prepared for the World Health Organization.

Grice, H.C., DaSilva, Stoltz, D.R., Munro, I.C., Clegg, D.J., and Abbatt, J.D. Testing of Chemicals for Carcinogenicity, Mutagenicity, Teratogenicity.

Munro, I.C. 1974. Chemicals that Cause Food Poisoning. Proc. of Symposium on Food Poisoning and its Significance in the Food Service Industry. Department of National Health and Welfare.

Stavric, B, Lacombe, R., Munro, I.C., and Grice, H.C. 1973. Studies on Chemical Impurities in Commercial Saccharin (Interim Report). Submitted to NRC Committee on Artificial Sweeteners of the National Academy of Sciences of the United States.

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Munro, I.C., Moodie, C.A., and Grice, H.C. 1973. An Evaluation of the Carcinogenicity of Commercial Saccharin. Submitted to NRC Committee on Artificial Sweeteners of the national Academy of Sciences of the United States.

Munro, I.C., Charbonneau, S.M., and McKinley, W.P. 1973. Studies on the Toxicity of Methylmercury. Commission of the European Communities, Luxembourg.

Grice, H.C., DaSilva, T.,Stoltz, D.R., Munro, I.C., Clegg, D.J., and Abatt, J.D. 1973. Testing of Chemicals, Mutagenicity and Teratogenicity. Department of National Health and Welfare.

Munro, I.C., Hasnain, S., Salem, F.A., Goodman, T., Grice, H.C., and Heggtveit, H.A. 1972. Cardiotoxicity of Brominated Vegetable Oils. Myocardiology Volume I. Recent Advances in Studies on Cardiac Structure and Function. p 588.

Dec-02

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18. CV Pariza

Appendix 18

MICHAEL W. PARIZA - CURRICULUM VITAE

Academic Rank: Director, Food Research Institute; Wisconsin Distinguished Professor and Chair, Department of Food Microbiology and Toxicology.

Education:

Ph.D.	Kansas State University	1973	Microbiology
M.Sc.	Kansas State University	1969	Microbiology
B.Sc.	University of Wisconsin-Madison	1967	Bacteriology

Professional Experience:

- Kansas State University: Graduate Teaching Assistant, Graduate Research Assistant, 1967-1969.
- U.S. Army (Armed Forces Institute of Pathology, Washington, D.C.): Biological Sciences Assistant, 1969-1971.
- Kansas State University: Graduate Teaching Assistant, Graduate Research Assistant, 1971-1973.
- The University of Wisconsin-Madison (McArdle Laboratory for Cancer Research): NIH Postdoctoral Traineeship with Professor Van R. Potter, 1973-1976.
- The University of Wisconsin-Madison (The Food Research Institute, Department of Food Microbiology and Toxicology): Assistant Professor, 1976-1981; Associate Professor, 1981-1984; Professor, 1984-Present; Associate Department Chair, 1981-1982; Department Chair, 1982-Present; Director, Food Research Institute, 1986-present.
- Wisconsin Distinguished Professor, 1993-present.
- Affiliate appointments at the University of Wisconsin: Wisconsin Comprehensive Cancer Center; Environmental Toxicology Center; Department of Nutritional Sciences; Department of Food Science.

Membership in Scientific Organizations:

The American Association for Cancer Research
The American Society for Microbiology
The American Association for the Advancement of Science
The Society of Sigma Xi
The Institute of Food Technologists
The Toxicology Forum
FASEB (The American Institute of Nutrition; The American Society for Clinical Nutrition)
The American Chemical Society
The American Oil Chemists' Society

Special Professional Recognition (1996-2002):

- Member, 1993 and 1998 Program Planning Committees, American Association for Cancer Research.
- Member, Institute of Medicine's Food Forum, 1993-1998.

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- Member, Board of Trustees, International Life Sciences Institute – North America, 1986-present.

- Member, National Academy of Sciences Committee on Comparative Toxicity of Naturally Occurring Carcinogens, 1993-1996.
- Testified at a hearing on "The Need for FDA Reform" held by the Subcommittee on Health and Environment, Committee on Commerce, U.S. House of Representatives, Washington, D.C., February 27, 1996.
- Invited participant (presented 2 lectures) at a symposium on conjugated linoleic acid sponsored by the American Oil Chemists Society, April 29, 1996, in Indianapolis.
- Invited participant and session moderator at a Workshop on Individual Fatty Acids and Cancer sponsored by the International Life Sciences Institute-North America, June 4-5, 1996, Washington, D.C.
- Recipient of 1996 Marqueta C. Huyck Endowed Lectureship, Wayne State University, Detroit, MI. Lecture title, "CLA: The fat that reduces obesity."
- Invited participant at an international symposium, "Fundamentals of Cancer Prevention," sponsored by the Princess Takamatsu Cancer Research Fund, Tokyo, Japan, November, 1996.
- Speaker at February 1997 dinner meeting of the Chicago Section of the Institute of Food Technologists, Chicago, IL.
- Lecturer at 1997 symposium series, "Modern Views in Nutrition," Iowa State University, Ames, IA.
- Chair of Symposium on "Enhancing the Regulatory Approval Process for Food Ingredient Technologies," sponsored by the National Academy of Sciences and the Institute of Medicine (Food and Nutrition Board, Food Forum), May 6-7, 1997.
- Elected Institute of Food Technologists Fellow, 1997.
- Invited lecture at 6th International Congress on Clinical Nutrition, at Banff, Alberta, Canada, July, 1997.
- Invited lecture at 16th International Congress of Nutrition, Montreal, Canada, July, 1997.
- Chair of Organizing Committee for the first CLA Forum, held at the University of Wisconsin-Madison, August, 1997 (highlights published in INFORM 9:69-73, 1998).
- Invited lecture at symposium sponsored by Best Foods, New Jersey, September, 1997.
- Invited lecture at Michigan State University, October, 1997.
- Invited lecture at the Japanese National Cancer Center, January, 1998.
- Invited lecture at the University of Illinois, February, 1998.
- Invited lecture, "Functional Foods: Technology, Functionality and Health Benefits," at Experimental Biology 98 symposium held in San Francisco, CA sponsored by ILSI (International Life Sciences Institute), April, 1998.
- Invited lecture on conjugated linoleic acid research for the Board of the Finnish Food Foundation, Helsinki, Finland, May, 1998.
- Two invited lectures at the Finnish Food Congress in Helsinki, Finland: "Food Safety: Risks and Management" (keynote address), and *E. coli* O157:H7 research at FRI, May, 1998.
- Invited lecture on conjugated linoleic acid at the National Cancer Institute, Bethesda, MD, May, 1998.

- Chair of session, "Modification of Cancer Risk by Inulin and Oligofructose," at conference entitled "Nutritional and Health Benefits of Inulin and Oligofructose" held at NIH (National Institute of Health) in Bethesda, MD, May, 1998.

- Invited lecture on conjugated linoleic acid at The Toxicology Forum, Aspen, CO, July, 1998.
- Invited lecture on conjugated linoleic acid at a symposium at Iowa State University, Ames, IA, September, 1998.
- Invited lecture on conjugated linoleic acid at a symposium sponsored by the Society of Toxicology, Reston, VA, October, 1998
- Co-organizer of the first "International Workshop on Conjugated Linoleic Acid Analysis," held in Washington, DC, January, 1999.
- Invited lecture on conjugated linoleic acid at the 1999 annual meeting of the American Oil Chemists Society.
- Invited lecture on conjugated linoleic acid at the 1999 annual meeting of the Institute of Food Technologists (IFT).
- Invited lecture on conjugated linoleic acid at the 40th International Conference on the Biochemistry of Lipids, Dijon, France, September, 1999.
- Invited lecture on conjugated linoleic acid at the 1999 Steenbock Symposium.
- Invited lecture at American Chemical Society conference on conjugated linoleic acid, August 2000.
- Featured speaker at American Chemical Society symposia on dietary supplements and functional foods for science writers, August 2000 and January 2001.
- Featured speaker at University of Wisconsin-Madison alumni fundraiser, held near Orlando, Florida in March 2001.
- Presented 2 invited lectures at the Food and Drug Administration in Washington, DC, April 2001.
- Invited participant at international conference on conjugated linoleic acid held in Alesund, Norway, June 2001.
- Summary speaker at food bioterrorism conference, organized by the International Life Sciences Institute-North America, held in Washington, DC, December 2001.
- Invited participant and speaker at a workshop on conjugated linoleic acid, organized by the National Institutes of Health, held in Bethesda, MD, May 2002.

Research Focus:

Conjugated linoleic acid

Patents:

1. Pariza, M.W., and Ha, Y.L. Methods of preventing oxidation, quenching singlet oxygen, and inhibiting mold growth and novel compositions thereof. U.S. 5,017,614

2. Pariza, M.W., and Ha, Y.L. Methods of chelating metal and novel compositions therefor. U.S. 5,070,104

3. Pariza, M.W., and Ha, Y.L. Octadecadienoic phospholipid esters, antioxidant and mold inhibiting compositions. U.S. 5,208,356

4. Cook, M.E., and Pariza, M.W. Methods for preventing weight loss, reduction in weight gain, and anorexia due to immune stimulation. U.S. 5,430,066

5. Cook, M.E. and Pariza, M.W. Method for increasing the efficiency of feed conversion in animals. U.S. 5,428,072

6. Cook, M.E., Pariza, M.W., Lee, K.N., and Wentworth, B.C. Method for controlling bird populations. U.S. 5,504,114.

7. Cook, M.E., Pariza, M.W., and Park Y. Method for reducing body fat in animals. U.S. patent application. U.S. 5,554,646.

8. Cook, M.E., Pariza, M.W., Yang, X., and DeVoney, D. Methods of treating animals to maintain or increase CD-4 and CD-8 cell populations. U.S. 5,674,901.

9. Cook, M.E., and Pariza, M.W. Dietetic foods containing conjugated linoleic acids. U.S. 5,760,082.

10. Cook, M.E., Cook, E.B., Stahl, J.L., Graziano, F.M., and Pariza, M.W. Methods of attenuating the allergic response in animals. U.S. 5,585,400.

11. Cook, M.E. and Pariza, M.W. Methods of treating animals to maintain or enhance bone mineral content and compositions for use therein. U.S. 5,804,210.

12. Satter, L.D., Dhiman, T.R., and Pariza, M.W. Method of increasing the CLA content of cow's milk. U.S. 5,770,247.

13. Cook, M.E., Pariza, M.W., and Jerome, D.L. Use of CLA to reduce the incidence of valgus and vargus leg deformities in poultry. U.S. 5,760,083.

14. Pariza, M.W. and Yang, X. Method of producing conjugated fatty acids. U.S. 5,856,149.

15. Pariza, M.W. and Lee, K.N. Method to reduce secretion of apolipoprotein ∃. U.S. 5,837,733.

16. Cook, M.E., Pariza, M.W., Kim, S., and DeVoney, D. Methods of treating animals to enhance natural killer lymphocyte function. U.S. 5,914,346.

17. Cook, M.E., Park, Y., and Pariza, M.W. Method for controlling body fat and/or body weight in animals and pharmaceutical compositions for use therein. U.S. 5,855,917.

18. Cook, M.E., Jerome, D.L., Pariza, M.W., and Buege, D.R. Method for increasing fat firmness and improving meat quality in animals. U.S. 5,851,572.

19. Cook, M.E. and Pariza, M.W. Method for maintaining an existing level of body fat and/or body weight. U.S. 5,814,663.

20. Cook, M.E. Pariza, M.W., Yang, X., and DeVoney, D. Methods of treating animals to maintain or increase CD-4 and CD-8 cell populations. U.S. 5,827,885.

21. Cook, M.E., Jerome, D., and Pariza, M.W. Method for selectively altering body fat level, feed efficiently, or weight gain. U.S. 6,020,378.

22. Pariza, M.W. and Yang, X. Methods of treating animals to maintain or increase CD-4 and CD-8 cell populations. U.S. 6,020,376.

23. Pariza, M.W. and Yang, X. Method of producing conjugated fatty acids. U.S. 5,856,149.

24. Pariza, M.W. and Yang, X-Y. Method of producing conjugated fatty acids. U.S. 6,060,304.

25. Cook, M.E., Jerome, D.L., Pariza, M.W., Buege, D.R., and Mozdziak, P. Method of increasing fat firmness and improving meat quality in animals. U.S. 6,060,087.

26. Cook, M.E., Whigham, L.D., and Pariza, M.W. Selective inhibition of cyclooxygenase-2. U.S. 6,077,868.

27. Cook, M.E., Aydin, R., and Pariza, M.W. Eggs enriched with conjugated linoleic acid. U.S. 6,113,973.

28. Cook, M., Yang, M., and Pariza, M.W. Method of increasing longevity and preventing body weight wasting in autoimmune disease by using conjugated linoleic acid. U.S. 6,395,782.

-- (Note: Foreign patent equivalents on each of these have issued or are pending.) --

Publications:

1. Pariza, M. W. and Iandolo, J. J. 1969. Coagulase production by injured *Staphylococcus aureus* MF-31 during recovery. *Appl. Microbiol.* 17:836-838.

2. Rosenthal, L. J., Martin, S. E., Pariza, M. W. and Iandolo, J. J. 1972. Ribosome synthesis in thermally shocked cells of *Staphylococcus aureus*. *J. Bacteriol.* 109:243-249.

3. Pariza, M. W. and Iandolo, J. J. 1974. Base ratio and DNA homology studies on six *Staphylococcus aureus*. *Appl. Microbiol.* 27:317-323.

4. Pariza, M. W. and Iandolo, J. J. 1974. Determination of genome size of selected bacteriophages of *Staphylococcus aureus*. *Appl. Microbiol.* 28:510-512.

5. Pariza, M. W., Becker, J. E., Yager, J. D., Jr., Bonney, R. J. and Potter, V. R. 1974. Enzyme induction in primary cultures of rat liver parenchymal cells, pp. 267-284. In W. Nakahara, T. Ono, T. Sugimura and H. Sugano (eds.) *Differentiation and control of malignancy of tumor cells*. University of Tokyo Press, Tokyo, Japan.

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145. Pariza, M. W. and Johnson, E. A. 2001. Evaluating the safety of microbial enzyme preparations used in food processing: Update for a new century. *Regulat. Toxicol. and Pharmacol.* 33:173-186.

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secretion from 3T3-L1 adipocytes. *Biochem. Biophys. Res. Commun.* 287:377-382.

150. Park, Y. and Pariza, M. W. 2001. The effects of dietary conjugated nonadecadienoic acid on body composition in mice. *Biochim. Biophys. Acta* 1533:171-174.

151. Park, Y. and Pariza, M. W. 2001. Lipoxygenase inhibitors inhibit heparin-releasable lipoprotein lipase activity in 3T3-L1 adipocytes and enhance body fat reduction in mice by conjugated linoleic acid. *Biochim. Biophys. Acta* 1534:27-33.

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153. Peters, J. M., Park, Y., Gonzalez, F. J. and Pariza, M. W. 2001. Influence of conjugated linoleic acid on body composition and target gene expression in peroxisome proliferator-activated receptor ∀-null mice. *Biochim. Biophys. Acta* 1533:233-242.

154. Evans, M., Park, Y., Pariza, M., Curtis, L., Kuebler, B., and McIntosh, M. 2001. *trans*-10,*cis*-12 conjugated linoleic acid reduces trigylceride content while differentially affecting peroxisome proliferator activated receptor (2 and aP2 expression in 3T3-L1 preadipocytes. *Lipids* 36:1223-1232.

155. Whigham, L. D., Higbee, A., Bjorling, D. E., Park, Y., Pariza, M. W., and Cook, M. E. 2002. Decreased antigen-induced eicosanoid release in conjugated linoleic acid-fed guinea pigs. *Am. J. Physiol. Regulatory Integrative Comp. Physiol.* 282(4):R1104-R1112.

156. Pariza, M. W. 2002. Commentary: Conjugated linoleic acid may be useful in treating diabetes by controlling body fat and weight gain. *Diabetes Technol. Therap.* 4:335-338.

157. Choi, Y., Park, Y., Storkson, J. M., Pariza, M. W., and Ntambi, J. M. 2002. Inhibition of stearoyl-CoA desaturase activity by the *cis*-9, *trans*-11 isomer and the *trans*-10, *cis*-12 isomer of conjugated linoleic acid in MDA-MB-231 and MCF-7 human breast cancer cells. *Biochem. Biophys. Res. Commun.* 294:785-790.

158. Kim, M. R., Park, Y., Albright, K. J., and Pariza, M. W. 2002. Differential responses of hamsters and rats fed high-fat or low-fat diets supplemented with conjugated linoleic acid. *Nutr. Res.* 22(6):715-722.

8/5/02

Appendix 19

CURRICULUM VITAE

Robert Andrew Roth

BUSINESS ADDRESSES:

Department of Pharmacology and Toxicology
B346 Life Sciences Building
Michigan State University
East Lansing, MI 48824

National Food Safety and Toxicology Center
Michigan State University
E. Lansing, MI 48824

PHONE: (517) 353-9841
E-MAIL: rothr@msu.edu

TRAINING:

1968	B.A. **Duke University**, Durham, NC Major: Chemistry
1975	Ph.D. **The Johns Hopkins University** School of Hygiene and Public Health Department of Environmental Medicine Baltimore, MD Major: Biochemical Toxicology
1977	Postdoctoral **Yale University** School of Medicine Departments of Anesthesiology and Pharmacology Field: Pulmonary Metabolism/Pharmacology

AWARDS:

PHS Predoctoral Traineeships, 9/68-9/69, 6/71-5/75
National Research Service Award: NIH Individual Postdoctoral Fellowship, 1976
Beecham Award for Research Excellence, College of Veterinary Medicine, Michigan State University, 1986
NIH MERIT Award, 1988
Honorary Member, Phi Zeta Honor Society of Veterinary Medicine, 1989
Michigan State University Distinguished Professor, 1991
Burroughs-Wellcome Toxicology Scholar Award, 1991
Kenneth DuBois Award, 2001 (given by the Midwest SOT for achievement in toxicology)

SOCIETY MEMBERSHIPS:

Society of Toxicology
American Society for Pharmacology and Experimental Therapeutics
American Association for the Advancement of Science
International Union of Pharmacology
International Society for the Study of Xenobiotics

000232

BOARD CERTIFICATION: Diplomate, American Board of Toxicology

PROFESSIONAL EXPERIENCE:

1968-69 1971-75	Graduate Student in Biochemical Toxicology The Johns Hopkins University Department of Environmental Medicine School of Hygiene and Public Health Baltimore, MD
1969-71	Toxicology Specialist Toxicology Division U.S. Army Environmental Hygiene Agency Edgewood Arsenal, MD
1975-77	Research Fellow in Pulmonary Pharmacology Yale University School of Medicine Departments of Anesthesiology and Pharmacology New Haven, CT **Mentor:** Dr. C. N. Gillis
1977-82	Assistant Professor Michigan State University Department of Pharmacology and Toxicology East Lansing, MI
1982-87	Associate Professor Michigan State University Department of Pharmacology and Toxicology and Center for Environmental Toxicology East Lansing, MI
1987-	Professor Michigan State University Department of Pharmacology and Toxicology and Institute for Environmental Toxicology East Lansing, MI
1995-	Associate Director for Toxicology Research National Center for Food Safety and Toxicology Michigan State University East Lansing, MI

TEACHING:

I deliver lectures to graduate students and professional students in three colleges. Courses in which I have participated that are directed to professional students include Principles of Pharmacology (allopathic and osteopathic human medical students, PHM 563), Principles of Pharmacology (veterinary students, PHM 556) and Veterinary Toxicology (VM 557). I have taught in the following courses offered to graduate students: Principles of Pharmacology and Toxicology (PHM 819), Cellular and Molecular Pharmacology and Toxicology (PHM820), Principles of Systemic and Integrative Pharmacology and Toxicology (PHM 821), Advanced General Pathology (PTH 857), Toxicologic Pathology (PTH850), Investigating the Lung (LCS 901). In addition, I have organized, moderated and lectured in Advanced Principles of Toxicology (PHM 814), a required course for the Institute for Environmental Toxicology doctoral program. I have organized and moderated a graduate seminar course on cellular effects of endotoxin (PHM921). Generally, topics taught in these courses include drug absorption, distribution,

pharmacokinetics, drug metabolism and excretion, general principles of pharmacology/toxicology, liver structure and function, liver and lung toxicology, oxygen radicals in disease, pulmonary metabolism and endothelial cell function, pulmonary hypertension.

I am or have been the major advisor for approximately 15 graduate students in the Ph.D. program in Pharmacology and Toxicology and have participated as a member of the thesis advisory committees of many students in other departments at MSU. In addition, I have been the major research advisor for three students in the Pathology Ph.D. program and one in masters program in the Department of Animal Science. Two of the students for whom I served as major thesis advisor have been awarded predoctoral fellowships from the Pharmaceutical Manufacturers Association Foundation. Four others were DVM postdoctoral research fellows, two of whom were awarded NIH individual postdoctoral fellowships (NRSA). Additionally, two others were awarded the Society of Toxicology (SOT) Hazelton Award and the SOT Proctor and Gamble Award for graduate students.

I have organized and moderated a Toxicology Journal Club within the Department of Pharmacology and Toxicology and a campus-wide informal pulmonary research discussion group. Numerous undergraduate students have performed independent study projects in my laboratory, and I have served as research advisor for several students in summer research programs at MSU (i.e., Biomedical Student Research Program for professional students, American Heart Association of Michigan program for undergraduates, NSF Summer Research Program for gifted high school students, Summer Undergraduate Research Fellowship program). I have lectured in the MSU Evening College course entitled "Fundamentals of Environmental Toxicology", which is taught to undergraduates interested in toxicology. In addition, I have participated in the Michigan Summer Institute for Gifted High School Students and a program to introduce high school students from lower income families to the biomedical sciences.

SERVICE RESPONSIBILITIES:

Institutional

Member, Curriculum Committee, College of Veterinary Medicine, 1977-1979.
Recording Secretary, Department of Pharmacology and Toxicology, 1977-1979.
Member, Student Performance Committee, College of Veterinary Medicine, 1980-1982.
Member, Safety Committee, Department of Pharmacology and Toxicology, 1980-1984. Chairman, 1980-1982.
Chairperson, Committee for Scheduling of Courses for Center for Environmental Toxicology, 1981-1982.
Member, Application Review Committee, Biomedical Student Research Program (Intramural), 1981.
Member, Application Review Committee, Biomedical Research Support Grants (Intramural), College of Human Medicine, 1981.
Member, Committee on Graduate Studies and Research, College of Veterinary Medicine, 1981-1983, 1988-1992, 1996-2000.
Member, College Advisory Council, College of Veterinary Medicine, 1981-1985; Vice-Chairperson, 1983-1984; Chairperson, 1984-1985.
Member, Admissions and Policy Committee for Graduate Program of the Center for Environmental Toxicology, MSU, 1982-1984.
Member, Search Committee for Faculty Position, Department of Pharmacology and Toxicology, 1982.
Member, Graduate Studies Committee, Department of Pharmacology and Toxicology, 1982-1983, 1990-1992.
Member, Search Committee for Dean of College of Veterinary Medicine, 1983-1984.
Member, Curriculum Committee, Department of Pharmacology and Toxicology, 1983-1984, 1985-1986.
Chairperson, Admissions and Policy Committee for Graduate Program in Environmental Toxicology, Institute for Environmental Toxicology, MSU, 1984-present.

Co-Organizer, Seminar Program, Department of Pharmacology and Toxicology, 1985-1986.
Member, Committee on Rosenberg Chair in the Biological Sciences, 1987.
Member, University Research Council, Michigan State University, 1988-1995.
Organizer, Seminar Program, Department of Pharmacology and Toxicology, 1988; Spring, Fall, 1990; Winter, Spring, 1992; Spring, 1994, Spring 1996.
Cochair, Toxicology Planning Committee, Food Safety and Toxicology Center, 1991-1996.
Chairperson, Search Committee for Faculty, Departments of Pharmacology and Toxicology and of Pathology, 1992.
Member, Student Performance Committee, College of Veterinary Medicine, 1992-1996.
Member, Dean's Design Team (for strategic planning process) , College of Veterinary Medicine, 1996-1997.
Member, Executive Committee, Pharmacological Sciences Training Grant, 1999-2001.
Member, Search Committee for Director, National Food Safety and Toxicology Center, 1999-2000.
Member, Search Committee for Genomics Faculty, 1999-2000.
Organizer, Joint NFSTC/IET Seminar Program, 1999-2000.
Member, Strategic Planning Committee, Dept. of Pharmacology and Toxicology, 2000-2001.
Member, Steering Committee for Food, Nutrition and Chronic Disease Fund, 1999-present.
Member, Advisory Committee, National Center for Food Safety and Toxicology, 1999-present.
Member, Biomedial Research Advisory Committee (advises Provost and VP for Research and Graduate Studies), 2000-present.
Member, Strategic Planning Committee, Institute for Environmental Toxicology, 2000-present.
Member, Graduate Committee, Dept. of Pharmacology and Toxicology, 2000-present.
Member, College Advisory Council, 2001-present.
Member, Steering Committee, Doctoral Program in Comparative Medicine and Integrative Biology, 1998-present.
Member, Faculty Advisory Committee, Department of Pharmacology and Toxicology, 1978-79; 1982-83; 1984-85; 1986-90; 1992-1996, 1998-1999, 2001-present.

State/Regional

Consultant to Kalamazoo County on Environmental Toxicology matters, 1979.
Guest Lecturer in adult education program of Haslett Public Schools, Haslett, MI.
Organizer and Moderator of Eighth Annual Pharmacology Research Colloquium, a regional student research conference, June 20, 1980.
Member, Tri-County Heart Unit Board of the Michigan Heart Association, 1980-1983.
Council Member, Michigan Regional Chapter of Society of Toxicology, 1983-1983.
Member, Research Grant Review Committee, American Heart Association of Michigan, 1983-1985.
Member, Steering Committee, The Multidisciplinary Asbestos/Mesothelioma/ Fibrous Dust Center, Lansing, MI, 1983-1987.
President, Board of Directors, Tri-County Unit, American Heart Association of Michigan, 1984-1985.
Chairperson of Scientific Session, 14th Annual Michigan Cardiovascular Research Forum, 9/84.
Member, Nominating Committee, Michigan Regional Chapter, Society of Toxicology, 1993, 1997.

National/International

Member, Advisory Panel for Evaluation of the NIEHS Environmental Toxicology Training Program, 1979-1982.
Chairperson of Scientific Session at Annual Meeting of Society of Toxicology (SOT), 1982, 1986, 1987, 1988, 1990.
Chairperson of Scientific Session at Joint Meeting of American Society of Pharmacology and Experimental Therapeutics and Society of Toxicology, Fall, 1982.

Research Grant Reviewer for The Johns Hopkins Center for Alternatives to Animal Testing, 1982-1987.
Special Reviewer, Toxicology Study Section, N.I.H., 2/84.
Outside Reviewer, Toxicology Study Section, N.I.H., 6/84.
Outside Reviewer, Toxicology Study Section, N.I.H., 9/84.
Member, Special Study Section, NHLBI, 6/84, for review of grant applications regarding "Markers for injury and repair of pulmonary endothelium."
Outside Reviewer, Toxicology Study Section, N.I.H., 2/85.
Special Reviewer, Toxicology Study Section, N.I.H., 6/85.
Member, Animals in Research Committee, Society of Toxicology, 1984-1986.
Member, Special Study Section, N.I.H., 10/88.
Member, Toxicology Study Section, N.I.H., 1985-1989.
Associate Editor, Toxicology and Applied Pharmacology, 1986-1993.
Member, Expert Panel on Pulmonary Toxicology, Committee on Biological Markers, National Academy of Sciences, 1986-1988.
Member, Program Committee, Society of Toxicology, 1988-1990.
Mechanisms Specialty Section, Society of Toxicology -- Member, Nominating Committee, 1983-84; Councillor, 1986-88; Vice-President-elect, 1988-89; Vice-President, 1989-90; President, 1990-91; Councillor, 1991-92.
Member, Advisory Council, Center for Alternatives to Animal Testing, 1988-1995.
Member, Education Committee, Society of Toxicology, 1990-1993.
Member, NIH Site Visit Team, NHLBI, 1990.
Associate Editor, Center for Alternatives to Animal Testing Newsletter, 1991-1995.
Member, Toxicology Program Review Committee, University of North Carolina, 1991.
Outside Reviewer, Toxicology Study Section, NIH, 1991.
Member, NIH Site Visit Team, NIEHS, 1991.
Symposium Organizer and Moderator, CAAT Symposium, 1991.
Organizer and Moderator, New Investigators Forum, Society of Toxicology Annual Meeting, 1992.
Reviewer, Special Study Section (Pharmacology), NIH, 2/92.
Consultant, Toxicology Program, Ponce School of Medicine, Ponce, PR, 1992-1996.
Reviewer, Special Study Section (Pharmacology), NIH, 10/92.
Reviewer, NIH Special Study Section (NIEHS, Ozone RFA), 1992
Consultant, Toxicology Program, University of Houston Health Sciences Center, 1992-1998.
Member, Executive Committee, Toxicology Division, ASPET, 1993-1995
Member, SOT Council, 1993-1995.
Member, Advisory Board, Southwest Center for Toxicology, University of Arizona, 1993-2000.
Member, Environmental Health Sciences Review Committee, NIEHS, 1994-1999.
External Member, Review Committee for Evaluation of Director, Curriculum in Toxicology, Univ. of North Carolina, 1997.
Outside Reviewer, Environmental Health Sciences Review Committee, NIEHS, 7/00.
Member, Board of Publications, Society of Toxicology, 1998-present; Chair, 1999-2001.
Member, Board of Directors, American Board of Toxicology, 1998-2002.
Reviewer, Ad Hoc, Environmental Health Sciences Review Committee, NIEHS, 2001.
Member, Editorial Board, Toxicology and Applied Pharmacology, 1984-1986; 1993-present.
Member, Editorial Board, Journal of Toxicology and Environmental Health, 1985-present.
Member, Editorial Board, General Pharmacology: The Vascular System, 1999-present.
Food Safety Specialty Section, Society of Toxicology-- Vice President Elect, 1999-2000; Vice President, 2000-2001; President, 2001-2002.

RESEARCH GRANTS FUNDED:

NIEHS Young Investigator Research Grant -- Environmental Agents and Non-respiratory Lung Function. 2/1/78-1/31/81; $88,000; Principal Investigator.

Michigan Heart Association Research Grant -- Pulmonary Biogenic Amine Disposition in Hypertension. 7/1/78-6/30/80, $22,000; Principal Investigator.

Council for Tobacco Research, Research Grant -- The Effects of Hypoxic Hypoxia, Carbon Monoxide and Treatments Influencing Response to Hypoxia on Endocrine-like Cells in Airways of Young Rabbits. 7/1/79-6/30/80, $14,000; Co-Investigator.

Michigan Lung Association Research Starter Grant -- Isolation, Identification and Toxicity of the Pulmonary Toxicant Paraquat and Its Pyrolysis Products. 12/1/78-11/30/79, $5,000; Co-Investigator.

College of Osteopathic Medicine Biomedical Research Support Grant (intramural) -- Pulmonary Biogenic Amine Disposition in Maturing Rats. 1/11/77-10/31/78, $2,000; Principal Investigator.

College of Osteopathic Medicine Biomedical Research Support Grant (intramural) -- Lung Clearance of Benzo(a)pyrene in Induced Rats. 12/1/78-2/28/80, $3,000; Principal Investigator.

College of Veterinary Medicine Biomedical Research Support Grant (intramural) -- Protection from Pulmonary Oxygen Toxicity. 10/1/79-9/30/80, $5,000; Principal Investigator.

College of Osteopathic Medicine Biomedical Research Support Grant (intramural) -- Effects of PBB and Monocrotaline on Lung and Liver. 12/1/79-11/30/80, $4,000; Principal Investigator.

Council for Tobacco Research, Research Grant -- The Effects of Hypoxic Hypoxia, Carbon Monoxide, and Treatments Influencing Hypoxia Toxicity on Endocrine-like Cells in Airways of Rabbit Fetuses. 7/1/80-6/30/82, $20,000; Co-Investigator.

Michigan Heart Association Research Grant -- Pulmonary Biogenic Amine Removal in Hypertension. 7/1/80-6/30/81, $15,000 (renewal); Principal Investigator.

U.S. Department of Agriculture -- Intestinal p-Cresol Production and Pulmonary Toxicity in the Young Pig. 9/1/81-8/31/83, $56,000; Co-Investigator.

NIEHS Research Grant -- Platelets and Lung Injury from Monocrotaline. 4/1/81-3/31/84, $97,000; Principal Investigator.

NHLBI Research Grant -- Blood Flow and Pulmonary Benzo(a)pyrene Disposition. 6/1/81-5/31/85, $143,000; Principal Investigator.

Michigan Heart Association Research Grant -- Diet and Cor Pulmonale from Monocrotaline. 7/1/83-6/30/84, $16,000; Principal Investigator.

Upjohn Company Gift -- Ibuprofen Effects on Development of Pulmonary Hypertension from Monocrotaline. $2,000, 1984.

Upjohn Company Grant -- Early Cardiopulmonary Effects of Monocrotaline Pyrrole: A Model of ARDS. 1/1/85-9/30/86, $6,000; Principal Investigator.

American Heart Association of Michigan -- Pulmonary Vascular Effects of ANTU. 7/1/85-6/30/86, $17,500; Principal Investigator.

Upjohn Company Grant -- 15-HETE in ARDS Models. 10/1/85-9/30/86, $10,800; Principal Investigator.

NIEHS Research Grant -- Platelets and Lung Injury from Monocrotaline (competing renewal). 4/1/84-3/31/88, $259,000; Principal Investigator.

NHLBI Research grant -- Effectors in Pulmonary Hypertension from Monocrotaline. 6/1/84-5/31/87, $260,000; Principal Investigator.

Upjohn Company Grant -- Studies of Mechanisms of Lung Injury. 10/1/86-9/30/87, $8,877; Principal Investigator.

NHLBI Research Grant -- Role of Eicosanoids in Airway Hyperresponsiveness. 10/1/86-9/30/90, $401,000; Co-Investigator.

NIEHS Research Grant -- Neutrophils and Hepatotoxicity. 6/1/86-5/31/92, $572,000; Principal Investigator.

College of Human Medicine Biomedical Research Support Grant (intramural) -- Hepatotoxic Agents and Neutrophil Chemotaxis. 7/1/88-6/30/89, $7,500; Co-Principal Investigator.

NIEHS Research Grant -- Platelets and Lung Injury from Monocrotaline (competing renewal). 4/1/88-3/31/93, $817,000; Principal Investigator. Converted to MERIT Award, 8/88 (funded through 3/98).

NIEHS Superfund Research and Education Program Project Grant -- Health Hazards from Groundwater Contamination. 1/1/89-11/30/91, Subproject 8, $634,000; Principal Investigator of Subproject.

NIEHS Research Grant -- Neutrophils and Hepatotoxicity. 12/1/91-11/30/96, $660,000; Principal Investigator.

NIEHS Superfund Research and Education Program Project Grant -- Health Hazards from Groundwater Contamination. 4/1/92-3/31/95, Suproject 5, $622,000; Principal Investigator.

NIEHS Research Grant -- Platelets and Lung Injury from Monocrotaline (MERIT Award). 4/1/93-3/31/98, $748,141; Principal Investigator.

NIEHS Superfund Research and Education Program Project Grant -- HealthHazards from Groundwater Contamination. 4/1/92-3/31/00, Suproject 4a , $320,000; Co-investigator.

NIDDK, NIH Research Grant--Distal Events in the Hepatotoxicity of Endotoxin. 7/1/96-6/30/00, $640,000; Principal Investigator.

Dow Foundation, SPHERE Program-Unrestricted research funds to National Food Safety and Toxicology Center. 11/14/97, $75,000; Principal Investigator.

NIEHS Research Grant -- Neutrophils and Hepatotoxicity. 4/1/98-3/31/03, $625,000; Principal Investigator.

Pharmacia Corp. Grant–Inflammation as a Determinant of Susceptibility to Drug Toxcicity: Toward an Animal Model of Drug Idiosyncrasy. 10/1/01-9/30/02, $115,000; Principal Investigtor.

MAJOR ADVISOR FOR THE FOLLOWING DOCTORAL STUDENTS:

Name: (b)(6)
Degree/Year of Graduation: PhD, 1982, Pharmacology and Toxicology
Thesis Title: Organ metabolic clearance: Role of flow, enzymic capacity, and binding in the metabolic clearance of benzo(a)pyrene by rat liver and lung
Current Position: Senior Associate, Commercialization Group, Research Corporation Technologies, Tucson, AZ

Name: (b)(6)
Degree/Year of Graduation: PhD, 1983, Pharmacology and Toxicology
Thesis Title: The role of platelets in monocrotaline-induced pulmonary hypertension
Current Position: Director, Toxicology, Chiron Corp., Seattle, WA

Name: (b)(6)
Degree/Year of Graduation: PhD, 1985, Pharmacology and Toxicology
Thesis Title: The role of immune effectors in monocrotaline pyrrole-induced pulmonary injury
Current Position: President, The Gillette Co., Gillette Medical Evaluation Laboratory, Needham, MA

Name: (b)(6)
Degree/Year of Graduation: PhD, 1986, Pharmacology and Toxicology/Environmental Toxicology
Thesis Title: The role of the platelet and platelet mediators in the pulmonary hypertensive response to monocrotaline pyrrole
Current Position: Associate Professor, Pharmacology & Toxicology, Michigan State University

Name: (b)(6)
Degree/Year of Graduation: PhD, 1988, Pharmacology and Toxicology
Thesis Title: Involvement of active oxygen metabolites and thromboxanes in phorbol myristate acetate toxicity to the isolated, perfused rat lung
Current Position: Consulting Toxicologist, Writeway Scientific Co., Nashville, TN

Name: (b)(6)
Degree/Year of Graduation: PhD, 1989, Pathology/Environmental Toxicology
Thesis Title: Monocrotaline pyrrole-induced pulmonary hypertension: Effects on pulmonary vasculature in vivo and in vitro
Current Position: Senior. Pathologist, Pfizer Corp., Ann Arbor, MI

Name: (b)(6)
Degree/Year of Graduation: PhD, 1990, Pharmacology and Toxicology/Environmental Toxicology
Thesis Title: Causal roles for neutrophils and glutathione in the liver injury caused by α-naphthylisothiocyanate
Current Position: Manager, Toxicological Sciences, Drug Safety Evaluation, Pfizer Corp., Drug Safety Evaluation, Groton, CT

Name: (b)(6)
Degree/Year of Graduation: PhD, 1991, Pharmacology and Toxicology
Thesis Title: The role of inflammatory mediators in endotoxin hepatotoxicity
Current Position: Assistant Professor, Pharmacology, Univ of Connecticut, Farmington, CT

Name: (b)(6)
Degree/Year of Graduation: PhD, 1992, Pharmacology and Toxicology/Environmental Toxicology

Thesis Title: Effects of monocrotaline pyrrole on cultured endothelial cell function: Potential role of endothelial changes in vascular remodeling and the development of pulmonary hypertension
Current Position: Officer, Institutional Animal Care & Use, Pharmacia, Kalamazoo, MI

Name: (b)(6)
Degree/Year of Graduation: PhD, 1992, Pathology/Environmental Toxicology
Thesis Title: *The role of the hemostatic system in monocrotaline pyrrole-induced* pneumotoxicity and pulmonary hypertension
Current Position: Senior Pathologist, Eli Lilly Corp., Greenville, IN

Name: (b)(6)
Degree/Year of Graduation: PhD, 1994, Pharmacology and Toxicology/Environmental Toxicology
Thesis Title: Mediators of inflammation and α-naphthylisothiocyanate hepatotoxicity
Current Position: Research Associate/Safety Pharmacology, Pfizer Global, Ann Arbor, MI

Name: (b)(6)
Degree/Year of Graduation: PhD, 1996, Pharmacology and Toxicology/Environmental Toxicology
Thesis Title: The role of platelets in lipopolysaccharide-induced liver injury
Current Position: Toxicologist, Michigan State Police, East Lansing, MI

Name: (b)(6)
Degree/Year of Graduation: PhD, 1997, Pathology/Environmental Toxicology
Thesis Title: The effect of monocrotaline pyrrole on cell proliferation and DNA synthesis in vitro and in vivo
Current Position: Toxicologic Pathologist, Sierra Biomedical, Inc., Dept. of Pathology, Sparks, NV

Name: (b)(6)
Degree/Year of Graduation: PhD, 1998, Pharmacology and Toxicology/Environmental Toxicology
Thesis Title: The role of thrombin in lipopolysaccharide-induced liver injury
Current Position: Scientist, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ

Name: (b)(6)
Degree/Year of Graduation: PhD, 1998, Pharmacology and Toxicology/Environmental Toxicology
Thesis Title: Characterization of impaired pulmonary neutrophil trafficking in the endotoxemic rat
Current Position: Assistant Professor, Veterinary Pathology, Michigan State University

CURRENT DOCTORAL STUDENTS:

(b)(6)

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PUBLICATIONS:

A. Research Publications in Peer-Reviewed Journals:

Roth, R.A. and Rubin, R.J.: Comparison of the effect of hypoxic hypoxia and of carbon monoxide: I. *In vivo* metabolism, distribution and action of hexobarbital. J. Pharmacol. Exp. Ther. 199: 53-60, 1976.

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NA- Not applicable