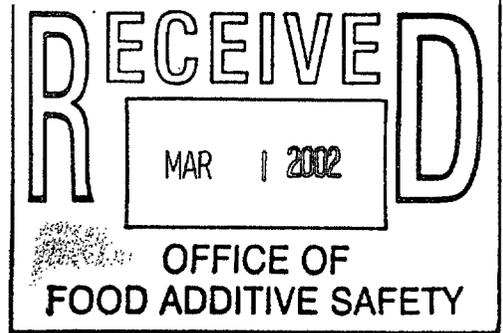


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Original Submission

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Pullulan GRAS Notification

Hayashibara International Inc.

8670 Wolff Court, Suite 200
Westminster, Colorado 80030
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Fax: 303-650-9860

Provided by:

Lee B. Dexter and Assoc.
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February 5, 2002

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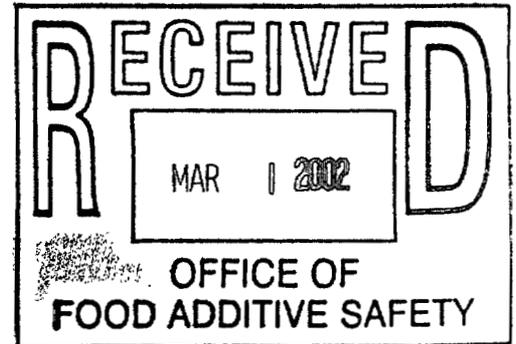
Letter

000021



HAYASHIBARA INTERNATIONAL INC.

8670 Wolff Court, Suite 200
Westminster, Colorado USA 80031-6953
Ph: 303-650-4590 Fax: 303-650-9860



February 13, 2002

Dr. Linda Kahl
Office of Food Additive Safety
Center for Food, Safety and Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Dear Dr. Kahl:

GRAS Notification for Hayashibara Pullulan

In accordance with the proposed rule for Substances Generally Recognized as Safe, which was published in the *Federal Register* at Vol. 62, No. 74 on April 17, 1997, Hayashibara International Inc. of Westminster, Colorado would like to submit notice of a claim that the use of Hayashibara Pullulan as a food ingredient is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, because such use is GRAS.

Pullulan is an extracellular polysaccharide, excreted by the polymorphic fungus *Aureobasidium pullulans*. The molecule is a linear α -D-glucan comprised of regular repeating trisaccharide units. The trisaccharide units are maltotrioses in which three glucose units are linked through 1,4-glucosidic bonds. Each maltotriose unit is terminally linked to a series of three other maltotrioses through α 1,6-glucosidic bonds creating a long stair-step-type structure. The molecular weight for commercial Pullulan may range from 8,000 to more than 2,000,000 daltons, depending upon the conditions under which the organism is grown.

A GRAS Report in support of the safe use of Pullulan in foods was prepared by Hayashibara International Inc. and Lee B. Dexter and Associates. The Report was reviewed by a Panel of Experts qualified by training and experience to assess the safety of food ingredients. The Experts concurred with Hayashibara International Inc.'s determination that Pullulan is safe for general use in foods. The Panel relied upon the results of numerous animal toxicology studies, a twenty-year history of safe consumption in Japan, the similarity of Pullulan to other GRAS food substances, such as amylopectin, and a large body of published literature. A copy of the Expert Opinion is attached to this notice.

000022



Page 2
February 12, 2002
Dr. Linda Kahl

This Notification of a claim for premarket exemption is based on a GRAS determination under proposed §170.36. Hayashibara International Inc. has prepared a Notification document in triplicate, which accompanies this letter. The Company would appreciate notice of the receipt of this document, and looks forward to any comments the agency would care to make on the Notification. If you have any questions regarding the content of the Notification, you may reach either myself at the number listed above or Lee B. Dexter at (512) 276-7408.

Sincerely,

A rectangular box with a red border, used to redact the signature of Alan B. Richards.

Alan B. Richards, Ph.D.
Vice President and General Manager

CC: Mr. Katsuaki Hayashibara, Hayashibara Company, Ltd.

000023

Claim

000024

Hayashibara International Inc. GRAS Notification

Introduction

Pullulan is a natural polysaccharide elaborated extracellularly by the fungal species *Aureobasidium pullulans*. It is commercially produced by a non-pathogenic and non-toxigenic strain of the organism utilizing corn syrup as the substrate. Pullulan has a linear structure comprised of maltotrioses in which three glucose units are linked through α -1,4-glucosidic bonds. The maltotrioses are in turn linked to a series of three other maltotrioses through α -1,6-glucosidic bonds creating a long stair-step-type structure (See Section II D). This type of molecular structure, in which α -1,4 and α -1,6-glucosidic bonds join various chain lengths of glucose is also found in such common food substances as the amylopectin fraction of corn and wheat starch, and in dextrans and maltodextrins. Due to its high molecular weight (50,000-500,000 daltons) and its bond configuration, Pullulan acts as a soluble dietary fiber in the human body.

The Hayashibara Company, Ltd. (Hayashibara) of Okayama, Japan developed the production strain of *Aureobasidium pullulans*, and the method for producing Pullulan more than two decades ago. Modern food processing research has shown that Pullulan may have an expanded role as a food ingredient.

Hayashibara is providing this Notification document to allow the FDA to evaluate whether the submitted notice provides a sufficient basis for a generally recognized as safe (GRAS) determination for Hayashibara Pullulan. The company believes that the document contains the information required in proposed § 170.36. The document is being submitted by Hayashibara International Inc., of Westminster, Colorado, which is a wholly-owned subsidiary of Hayashibara Company, Limited. Both companies will be referred to as "Hayashibara" in this Notification, unless a specific distinction is necessary.

In compliance with 21 CFR § 170.30, Hayashibara determined that Hayashibara Pullulan could be considered GRAS when used in accordance with current Good Manufacturing Practices. Hayashibara wishes to voluntarily notify the Center for Food Safety and Applied Nutrition (CFSAN) of that determination, and according to proposed § 170.36, the company is submitting the following GRAS exemption claim.

Hayashibara International Inc. has prepared a GRAS Report, which forms the basis for the information found in this Notification. The company commissioned a panel of experts (Expert Panel), qualified by scientific training and experience to assess the safety of food ingredients, to critically evaluate the Pullulan GRAS Report as well as other data and information relevant to the use and safety of this ingredient. As the result of various telephone conferences and a meeting held on July 25, 2001, the Expert Panel concurred with the company's determination that

Hayashibara Pullulan can be considered generally recognized as safe for general use in food. Based on the data and information contained in the Report and the opinion of the Expert Panel (which is attached to this notification), Hayashibara explicitly accepts responsibility for the GRAS determination of Hayashibara Pullulan.

Section I. GRAS Exemption Claim

Hayashibara International Inc. hereby notifies the U.S. Food and Drug Administration that the use of Hayashibara Pullulan as a food ingredient is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, because Hayashibara has determined that such use is GRAS.

1. Notifier:

Hayashibara International Inc.
8670 Wolff Court, Suite 200
Westminster, Colorado, USA 80030
Telephone: (303) 650-4590
Fax: (303) 650-9860

2. Common or Usual Name:

Pullulan

3. Applicable Conditions of Use:

Applications for Pullulan include general use in foods as a multiple-use direct additive. The ingredient should be used under conditions of current Good Manufacturing Practice.

The FDA has published a list of 32 physical or technical functional effects for which direct food ingredients may be added to food. These are codified at 21 CFR §170.3 (o) (1-32). The various physical and technical functional effects for Pullulan are covered under the following terms as listed under 21 CFR §170.3 (o).

- (8) "Emulsifiers and emulsifier salts": Substances, which modify surface tension in the component phase of an emulsion to establish a uniform dispersion or emulsion.
- (14) "Formulation aides": Substances used to promote or produce a desired physical state or texture in food, including carriers, binders, fillers, plasticizers, film-formers, and tableting aids, etc.
- (16) "Humectants": Hygroscopic substances included in food to promote retention of moisture, including moisture-retention agents and antidusting agents.
- (20) "Nutrient supplements": Substances which are necessary for the body's nutritional and metabolic processes.
- (24) "Processing aids": Substances used as manufacturing aids to enhance the appeal or utility of a food or food component, including clarifying agents, clouding agents, catalysts, flocculants, filter aids, and crystallization inhibitors, etc.
- (28) "Stabilizers and thickeners": Substances used to produce viscous solutions or dispersions, to impart body, improve consistency, or stabilize emulsions, including suspending and bodying agents, setting agents, jellying agents, and bulking agents, etc.
- (29) "Surface-active agents": Substances used to modify surface properties of liquid food components for a variety of effects, other than emulsifiers, but including solubilizing agents, dispersants, detergents, wetting agents, rehydration enhancers, whipping agents, foaming agents, and defoaming agents, etc.
- (31) "Synergists": Substances used to act or react with another food ingredient to produce a total effect different or greater than the sum of the effects produced by the individual ingredients.
- (32) "Texturizers": Substances, which affect the appearance or feel of the food.

4. Basis of the GRAS Determination

The basis of the GRAS determination for Hayashibara Pullulan was the use of scientific procedures.

5. Availability of Data and Information and Key to References

The data and information that are the basis of the GRAS determination for Hayashibara Pullulan will be available for FDA review and copying at the address of the notifier listed above. The notifier will also be pleased to provide the agency with a copy of the GRAS Report, or any references contained therein, upon written request. Throughout this Notification, citations to the published literature or other pertinent information, which were

included in the GRAS Report, are denoted as follows: [Author (*et al*), Year, Tab (number) Volume (number)]. In order to facilitate review of this document a complete list of references from the Pullulan GRAS Report is included in Appendix 2 as a key. Recently identified references, which were not included in the GRAS Report are shown between parentheses () within the text of this document and given in a standard bibliographic form.

6. Signature of an official for Hayashibara International Inc.

Official for Hayashibara International Inc.

Date



Feb. 05, 2002

Alan B. Richards, Ph.D.
Vice President and General Manager

Notification
Table of Contents

000029

Hayashibara International Inc.
Pullulan GRAS Notification

| Table of Contents | Page | Sec |
|---|-------------|------------|
| Section II. Chemical Identity | 1 | II |
| A. Common or Usual Name and Identity..... | 1 | II |
| B. Formal Names (IUPAC or Chemical Abstract Names)..... | 1 | II |
| C. Synonyms: Other Common Names, Trade Names..... | 1 | II |
| D. Chemical Formulae, Structures and Molecular Weights..... | 1 | II |
| E. Chemical Abstract Service Registry Number (CAS Registry No) | 2 | II |
| F. Description..... | 2 | II |
| G. Physical Properties..... | 3 | II |
| Table 1: Physical Properties and Characteristics of Pullulan | 3 | II |
| H. Raw Materials and Specifications..... | 4 | II |
| 1. Raw Materials Used in the Production of HBC Pullulan..... | 4 | II |
| 2. Raw Material Specifications | 5 | II |
| I. Production Process and Quality Controls | 5 | II |
| 1. Introduction | 5 | II |
| 2. The Hayashibara Process | 5 | II |
| Figure 1: Process Flow Diagram for Hayashibara Pullulan..... | 7 | II |

Table of Contents (Continued)

| | Page | Sec |
|--|-------------|------------|
| 3. Process Control Overview..... | 8 | II |
| 4. Manufacturing Process Controls..... | 9 | II |
| 5. Manufacturing Facilities and Equipment | 9 | II |
| 6. Packaging and Labeling..... | 10 | II |
| 7. Product Release Controls | 10 | II |
| J. Complaint and Recall Procedures..... | 10 | II |
| K. Multiple Products | 10 | II |
| L. HBC Pullulan Specifications, Product Identity, and Purity..... | 11 | II |
| 1. Product Specifications..... | 11 | II |
| Table 2: Final Product Specifications of HBC Pullulan PF-20..... | 12 | II |
| Table 3: Final Product Specifications of HBC Pullulan PF-10 | 13 | II |
| 2. Product Identity..... | 13 | II |
| 3. Analysis of 10 Lots | 14 | II |
| 4. Certificates of Analysis for Pullulan PF-10 | 14 | II |
| 5. Lead and Specific Heavy Metal Analyses..... | 15 | II |
| 6. Mycotoxin Levels..... | 15 | II |

Table of Contents (Continued)

| | Page | Sec |
|---|-------------|------------|
| Table 4: Certificates of Analyses for Pullulan PF-10..... | 16 | II |
| Table 5: Analytical Test Results of 10 Lots of HBC Pullulan PF-20 | 17 | II |
| Table 6: Analyses of Lead Levels in HBC Pullulan PI-20..... | 18 | II |
| Table 7: Analyses of Specific Heavy Metals in HBC Pullulan PF-20 and PI-20..... | 18 | II |
| Table 8: Mycotoxin Levels of Hayashibara Pullulan..... | 19 | II |
| 7. Antibiotic and Aureobasidin Production..... | 20 | II |
| Table 9: Antibacterial Activities in HBC Pullulan Products. | 21 | II |
| 8. Microbial Content of Commercial HBC Pullulan Products..... | 22 | II |
| Table 10: Microbial Analyses of Commercial HBC Pullulan Products | 23 | II |
| M. Test Methods | 24 | II |
| N. Stability of Pullulan..... | 24 | II |
| O. Explanation of the Lot Code | 24 | II |
| P. Summary | 25 | II |
| Section III. Technical Effects, Intended Uses, Consumption Estimates and Information on Self-Limiting Levels | 26 | III |
| A. Intended Uses, Functional Effects, and Use Levels | 26 | III |

Table of Contents (Continued)

| | Page | Sec |
|--|-------------|------------|
| 1. Intended Uses | 27 | III |
| 2. Physical and Technical Functional Effects and Use Levels..... | 28 | III |
| Table 11: Intended Use and Functional Effects | 29 | III |
| B. Consumption and Exposure Estimates | 31 | III |
| 1. Consumption | 31 | III |
| 2. Exposure Estimate..... | 31 | III |
| Table 12: Estimated Intake of Pullulan in Selected Food Categories..... | 33 | III |
| C. Self-Limiting Levels..... | 35 | III |
| D. Other Limiting Factors | 36 | III |
| Section IV. Safety | 37 | IV |
| A. Pullulan and Polyglucoses..... | 37 | IV |
| B. Commercial Pullulan..... | 39 | IV |
| C. Pathogenicity of the Source Organism. <i>A. pullulans</i> | 40 | IV |
| D. Acute Toxicity in Mice and Rats..... | 41 | IV |
| E. Long-term Feeding Study in Rats..... | 43 | IV |
| Table 13: Pullulan Intake | 46 | IV |
| Table 14: Organ Weights and Organ/Body Weight Ratios in Male Rats Fed for 62 Weeks..... | 48 | IV |

Table of Contents (Continued)

| | Page | Sec |
|--|-------------|-------------------|
| Table 15: Organ Weights and Organ/Body Weight Ratios in Female Rats Fed for 62 Week | 49 | IV |
| Table 16: Gross Examination and Histopathological Finds Relating to Pneumonia in Rats at the Termination of the Study..... | 50 | IV |
| Figure 2: Survival of Rats after a 62 Week Pullulan Intake Study..... | 53 | IV |
| F. Human Consumption Study | 54 | IV |
| G. History of Safe Use | 56 | IV |
| H. Fate of Pullulan in the Digestive Tract..... | 57 | IV |
| I. Systematic Exposure..... | 61 | IV |
| J. Mutagenicity of Pullulan | 62 | IV |
| K. Conclusions on Safety..... | 62 | IV |
| Appendices | | Appendices |
| Expert Opinions | | Appendix I |
| Expert Panel | | Appendix I |
| Joseph F. Borzelleca, Ph.D. | | |
| Michael W. Pariza, Ph.D. | | |
| Michael P. Doyle, Ph.D. | | |
| Cleve B. Denny, M.S. | | |
| Expert Opinions Requested by the Panel or the Sponsor | | |
| Donald G. Ahearn, Ph.D. and Libero Ajello, Ph.D..... | | Appendix I |
| George C. Fahey, Jr., Ph.D. September 17, 2001. | | Appendix I |
| George C. Fahey, Jr., Ph.D. December 21, 2001. | | Appendix I |

Table of Contents (Continued)

Page Sec

Complete List of References..... Appendix II

Hayashibara Pullulan Manufacturing Process..... Appendix III

Hayashibara Pullulan Manufacturing Process Controls..... Appendix IV

Section II

000036

Section II Chemical IdentitySection II: Chemical Identity of Pullulan

A. Common or Usual Name and Identity

Common Name: Pullulan, Pullulane (French)

Chemical family: Polysaccharide

B. Formal Names (IUPAC or Chemical Abstracts Names)

Chemical Abstracts Name: Pullulan

C. Synonyms; other Common Names, Trade Names

Other names: 1,4 -1,6- α -D-Glucan, 1, 6- α -linked maltotriose

Tradenames: HBC Pullulan

Pullulan PI-10

Pullulan PF-10

Pullulan PI-20

Pullulan PF-20

D. Chemical Formula, Structure and Molecular Weight

Chemical formula: $(C_6H_{12}O_5)_n$

Molecular weight: Approx. 200,000 (mean)

Chemical structure:

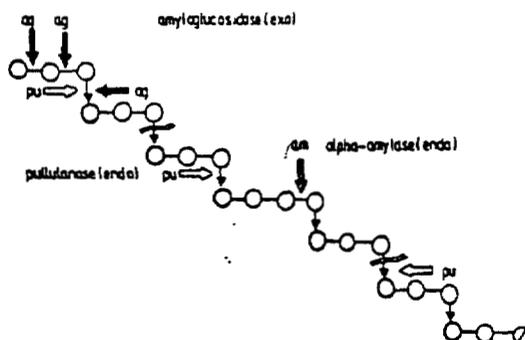
From: [Catley, *et al.*, 1986 Vol 2 Tab 5]

Fig. 1. The linear structure of pullulan, showing maltotriose residues with the occasional replacement by maltotetraose: O, α -D-Glc; —, (1 \rightarrow 4) linkages; and \downarrow , (1 \rightarrow 6) linkages. Typical sites of enzymic attack are shown by large arrows. Amyloglucosidase (ag) acts on both (1 \rightarrow 4) and (1 \rightarrow 6) linkages, sequentially from the non-reducing end; pullulanase (pu) acts randomly on (1 \rightarrow 6) linkages; and porcine α -amyrase (am) acts randomly on the terminal (1 \rightarrow 4) linkage of maltotetraosyl residues.

000037

Section II Chemical Identity

E. Chemical Abstracts Service Registry Number (CAS Registry No.)

CAS #: 9057-02-7

F. Description:

Pullulan is an extracellular polysaccharide, which is excreted by the polymorphic fungus *Aureobasidium pullulans* [Catley, *et al.*, 1986 Vol 2 Tab 5]. Structurally, it is a linear α -D-glucan comprised of regular repeating trisaccharide units. These maltotrioses (in which three glucose units are linked through α -1,4-glucosidic bonds) are in turn terminally linked to a series of other maltotrioses through α -1,6-glucosidic bonds creating a long stair-step-type structure (Section D. above). Alternatively, the structure may also be described as 6- α -D-glucosylmaltose linked by (1 \rightarrow 4) bonds [Catley, *et al.*, 1986 Vol 2 Tab 5]. The molecular weight range for Pullulan may range from 8,000 to more than 2,000,000 daltons, depending upon the conditions under which the organism is grown [Sugimoto, 1978 Vol 4 Tab 42, Ueda *et al.*, 1963 Vol 4 Tab 46, and Catley, *et al.*, 1986 Vol 2 Tab 5]. Hayashibara produces products of different molecular weights and specifications. PF is the designation for food grade, while PI is a more deionized product. Currently Hayashibara manufactures products with mean molecular weights of 100,000 and 200,000. Other molecular weight products can also be produced.

Pullulan is closely related to amylopectin, dextrin and maltodextrin, which have been affirmed GRAS under 21 CFR Part 184, in that all four substances consist exclusively of glucose units linked through α -1,4- and α -1,6-glucosidic bonds [LSRO, 1975 Vol 3 Tab 26]. Amylopectin is a major component of starch, and both maltodextrin and dextrans are prepared from starch [LSRO, 1975 Vol 3 Tab 26]. For comparison, maltodextrin, consists of approximately 20% α -1,6-glucosidic bonds, while Pullulan contains approximately 30% α -1,6-glucosidic bonds. A typical food starch, such as cornstarch, consists of 95% α -1,4-glucosidic bonds and 5% α -1,6-glucosidic bonds [Whistler, *et al.*, 1984 Vol 4 Tab 53, Sugimoto, 1978 Vol 4 Tab 42, and LSRO, 1975 Vol 2 Tab 26]. Catley, *et al.* reported that Pullulan may also contain a small percentage of maltotetraose units randomly distributed throughout the molecule in place

Section II Chemical Identity

of the maltotriosyl residues [Catley, *et al.*, 1986 Vol 2 Tab 5].

G. Physical Properties of Pullulan

Pullulan has various physical properties that can be used for food-associated applications. These properties may be useful in producing products that dissolve easily in aqueous environments and, are resistant to changes in viscosity with changes in pH, temperature or the use of salts. Additionally, Pullulan's film forming properties can be used to form films or coatings on foods, and oxygen barriers or matrixes to hold flavors and protect food quality. Pullulan is capable of being compressed into tablets, where its particular dissolution properties can be used to release a suspended or solubilized substance over time. It can also function as a binder or humectant. Table 1 contains a list of the physical properties of Pullulan that can be exploited for various food products.

Table 1
Physical Properties and Characteristics of Pullulan

| Physical Property | Characteristics |
|--------------------------|---|
| Solubility | 1) Highly soluble in cold or hot water. 2) Not soluble in organic solvents except dimethylformamide or DMSO. 3) Ether and ester substitution makes it insoluble in water and soluble in organic solvents. |
| Stability | 1) 1, 4 and 6 carbons are bound, making Pullulan non-reducing and relatively stable. 2) Decomposes and carbonizes at 250-280°C in a manner similar to starch. 3) Not volatile or exothermic. |
| Viscosity | 1) Dissolves in water producing a stable viscous solution. 2) Does not gel. 3) Viscosity proportional to molecular weight (Mw). 4) Low viscosity compared to other polysaccharides (gums). 5) Surface tension close to water (74 dyne/cm ²). 6) Maintains viscosity over large range of pH. 7) Maintain viscosity in presence of salts, most metal ions, especially sodium. 8) Heating at pH < 3 causes decreased viscosity, like other polysaccharides. 9) Heating high Mw Pullulan results in decreased viscosity, while lower Mw Pullulan does not decrease. |

Section II Chemical Identity

Table 1: Physical Properties and Characteristics of Pullulan (Cont'd)

| | |
|---|---|
| Film Forming | 1) Readily forms films with unique properties. 2) 5-25% aqueous solutions can be formed into edible film. 3) Low oxygen permeability (0.5 cc/m ² /24 hours at 60% RH and 23°C), thermally stable, anti-static, and retains elasticity after being frozen. 4) Dissolves faster than other film forming agents. 5) Holds flavors and is resistant to oils. |
| Adhesiveness and Binding Property | 1) Intensively adhesive. 2) Adheres to foods. 3) Good processing aid for pulverization and agglomeration. 4) Bond strength greater than oxidized starch, corn starch and phenol resin. |
| Moldability, Spinnability and Tabletability | 1) With moisture it is directly compressible under heat. 2) High surface hardness, elasticity, and draw ratio of 3-15%. 3) Forms tablets under low pressure. 4) Dissolves from surface. 5) Use as slow release vehicle. |
| Moisture Retention | 1) RH < 70% 10-15% moisture content. 2) Not hygroscopic or sticky. 3) Used as a humectant and binder. |
| Refractive Index | 1) Significant positive linear correlation of concentration and refractive index at 20 and 45°C |
| Biodegradability | 1) Indigestible polysaccharide. 2) Degraded by microbial enzymes pullulanase and isopullulanase. 3) Minor susceptibility to human digestive enzymes. 4) Fermented to short chain fatty acids by fecal bacteria. |

H. Raw Materials and Specifications**1. Raw Materials Used in the Production of HBC Pullulan**

The following substances are used in the manufacture of Pullulan:

- Ammonium Sulfate
- Beer Yeast Extract
- Beer Yeast Extract
- Calcium Hydroxide
- Caustic Soda
- Corn Syrup
- Diatomaceous Earth
- Diammonium Phosphate
- Dipotassium Phosphate
- GY Syrup (a corn syrup)
- Hydrochloric Acid
- Ion Exchange Resin
- Magnesium Sulfate
- Salt
- Silicone Oil
- Sodium Glutamate
- Zinc Carbon Chloride (activated charcoal)

Section II Chemical Identity

2. Raw Material Specifications

Food grade specifications for all raw material used in the manufacture of Pullulan were provided for the review of the Pullulan Expert Panel. Hayashibara Company, Ltd. analyzes all incoming raw materials periodically for compliance with their published specifications.

I. Production Process and Quality Controls

Pullulan has been sold into the food industry and eaten by consumers in Japan for more than 20 years. Hayashibara Company, Ltd. has continued to optimize the production of Pullulan. The general production process, possible variations, and methods to ensure the quality and safety of the products will be discussed in the following section.

1. Introduction

HBC Pullulan is produced by mesophilic (22°-30°C) fermentation of starch syrup with the black yeast, *Aureobasidium pullulans*. *Aureobasidium pullulans* is non-pathogenic and non-toxigenic, and is ubiquitous in nature. Various researchers have studied the characteristics and taxonomic position of this organism. A brief summary of these findings is included in Appendix 3. Hayashibara uses a strain of the organism that produces only small amounts of black-pigment, and grows rapidly to yield maximum quantities of Pullulan. Pullulan is elaborated extracellularly into the culture medium from which it is recovered and purified as described below [Catley, 1971 Vol 2 Tab 4].

2. The Hayashibara Process

The strain of *Aureobasidium pullulans* used for the production of Pullulan is labeled "Hayashibara strain". The organism and the particular strain are non-pathogenic and non-toxigenic, and are not the product of genetic engineering. To assure that a pure culture of the Hayashibara strain is used in Pullulan production, stock cultures are freeze-dried and stored in ampules. At the time of cultivation, stock cells are cultured from the ampules and streaked on agar plates. If, after colony formation, the purity

Section II Chemical Identity

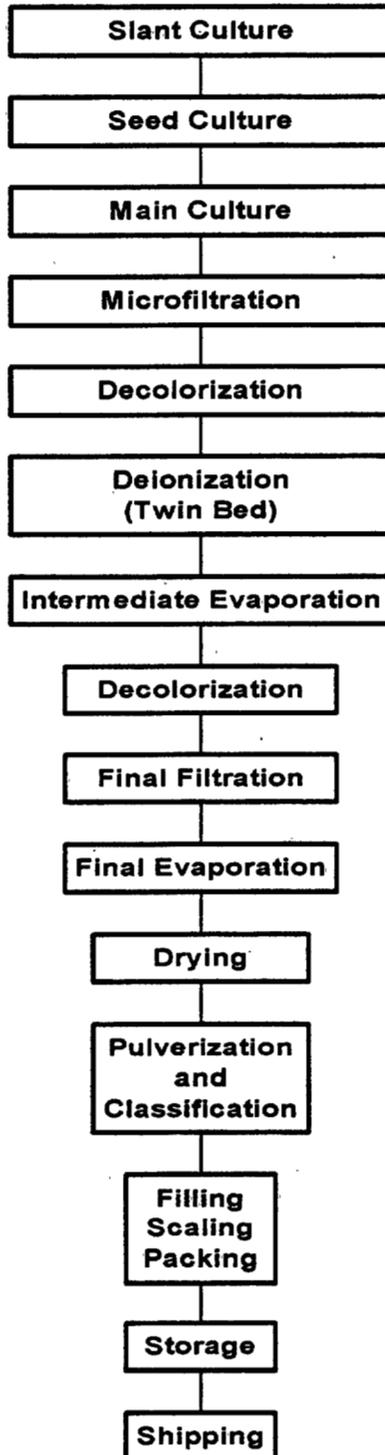
of the culture is confirmed, one colony is transferred to an agar slant. This colony is then used as the inoculum for the production of Pullulan.

A process flow diagram of the Hayashibara Pullulan production scheme is shown below. The black pigment produced by the Hayashibara strain is decolorized with activated carbon following pH adjustment of the culture medium.

Appendix 3 provides a further description of the production process.

Section II Chemical Identity

Figure 1
Process Flow Diagram
For Hayashibara Pullulan



Section II Chemical Identity

3. Process Controls Overview

HBC Pullulan is manufactured at Okayama Plant II. Quality control activities from several sections of the company are jointly responsible for Pullulan quality assurance. Each section has its own analytical laboratory within the plant, equipped with all the laboratory equipment normally required for the analytical control of raw materials and finished products. If necessary, the plant also has access to other laboratories within the company and the city of Okayama that are equipped to handle more complicated issues.

Analytical methods are designed to provide longitudinal data and information on the identity, purity, quality, strength, and stability of Pullulan. Feedback from the quality control laboratories to the manufacturing plant is used to adjust critical control points if necessary to maintain the desired properties and characteristics of the product. Such a system ensures that HBC Pullulan is manufactured under current Good Manufacturing Practice (cGMP), and that it will meet its published specifications.

Section II Chemical Identity

Tower and TBA Tower), and polyethylene (in the hydrochloride acid tank). These were designed and are used under cGMP.

6. Packaging and Labeling

Twenty-five kg of Pullulan is weighed into an anti-static polyethylene bag (thickness: 0.1 mm), and secured with a rubber band. The bag is placed in a cardboard box with a cardboard pad on the bottom. The box has a label printed with the appropriate information. The box is sealed with adhesive tape. The specifications for the packaging material were provided to and reviewed by the Expert Panel.

7. Product Release Controls

The Drug Additives Manufacturing Supervisor of Hayashibara Company, Ltd, releases Hayashibara Pullulan after a review of the raw materials and specification analyses, and comparison to final product specifications.

J. Complaint and Recall Procedures

There are detailed procedures to be followed if Hayashibara receives a complaint concerning product quality. These included a thorough investigation and reporting system, and if necessary a product recall.

K. Multiple Products

Hayashibara Pullulan is currently commercially available in two molecular weights. The products are designated as "10" and "20", which represent mean molecular weights of 100,000 and 200,000. There are also two specification grades for Pullulan that are called "PF" for food grade, and "PI" for a highly deionized product.

It is possible to predictably vary the molecular weight of the Pullulan produced by varying the dependent conditions of cultivation [Sugimoto, 1978 Vol 4 Tab 42]. The conditions include substrate concentration, temperature, pH, aeration and agitation rates.

Section II Chemical Identity**4. Manufacturing Process Controls**

Current Good Manufacturing Practices (cGMP) are used in the handling of raw materials, the production of the Pullulan, and the process controls. To guarantee the purity of the culture several steps are taken and critical control points are used. Briefly, all containers and culture media used for cultivation are thoroughly sterilized, and the air used for aeration of the culture is filtered. At regular intervals during fermentation, microscopic examination and pH determination of the culture, and analyses of the Pullulan are conducted to assure purity.

If a culture is contaminated, it is sterilized and discarded. The batch is not reprocessed. To the extent possible, the source of the contamination is determined, and appropriate counter measures are adopted to prevent recurrences.

After cultivation the live organisms of the Hayashibara strain are removed from the culture media by microfiltration. The Pullulan containing media is then sterilized with heat as an added measure of safety. The absence of the live strain of *A. pullulans* in the product is determined by culture. The Hayashibara strain exhibits characteristic growth morphology, and is therefore recognizable. For those colonies that are difficult to classify, inoculation into liquid medium and assessment of the colony's ability to produce Pullulan is determined.

Appendix 4 lists the process controls that are in place in the manufacture of Hayashibara Pullulan.

5. Manufacturing Facilities and Equipment

It should be noted that while the building is used for the production of other products, the Pullulan production area is separated from the other areas and dedicated for this purpose.

The materials that come in direct contact with Pullulan during the production include stainless steel, natural hard rubber linings (in the TBK

Section II Chemical Identity

The purpose for altering the molecular weight of the final product is to provide products whose viscosity potential matches an intended use. For example, a 10% solution of HBC PF-20 (mean molecular weight=200,000) has a viscosity of 100-180 mm²/s, whereas HBC PF-10 (mean molecular weight=100,000) has a viscosity of 15-25 mm²/s at the same concentration [Hayashibara, Internal Data, 2000 Vol 2 Tab 13]. The different viscosities are specified by product in the company's published specifications.

L. HBC Pullulan Specifications, Product Identity, and Purity**1. Product Specifications**

Final food grade product specifications have been developed for HBC Pullulan products. Tables 2 and 3 list the specifications for PF-20 and PF-10.

Section II Chemical Identity

Table 2
Final Product Specifications of HBC Pullulan PF-20

| Variable | Specification |
|--|--|
| Appearance | White to slightly yellowish powder, tasteless and odorless |
| Pullulan purity (dry basis) | > 90% |
| Loss on drying | < 6.0% |
| Residue on ignition | < 1.5% |
| Viscosity (10 wt%, 30°C) | 100 - 180 mm ² /s |
| Lead | < 0.1ppm |
| Arsenic | < 2 ppm |
| Heavy metals | < 5 ppm |
| pH | 5.0-7.0 |
| Mono, di- and oligosaccharides (dry basis) | < 10% |
| Coliforms | < 10/g maximum |
| Yeast and molds | < 100/g maximum |
| <i>Salmonella sp.</i> | Negative/25 g |
| <i>E. coli</i> | Negative/25 g |
| <i>Staphylococcus aureus</i> | Negative/25 g |

Section II Chemical Identity

Table 3
Final Product Specifications of HBC Pullulan PF-10

| Variable | Specification |
|--|--|
| Appearance | White to slightly yellowish powder, tasteless and odorless |
| Pullulan purity (dry basis) | > 90% |
| Loss on drying | < 6.0% |
| Residue on ignition | < 5.0% |
| Viscosity (10 wt%, 30°C) | 15-25 mm ² /s |
| Lead | < 0.1ppm |
| Arsenic | < 2 ppm |
| Heavy metals | < 5 ppm |
| pH | 5.0-7.0 |
| Mono, di- and oligosaccharides (dry basis) | < 10% |
| Coliforms | < 10/g maximum |
| Yeast and molds | < 100/g maximum |
| <i>Salmonella sp.</i> | Negative/25g |
| <i>E. coli</i> | Negative/25g |
| <i>Staphylococcus aureus</i> | Negative/25g |

2. Product Identity

HBC Pullulan occurs as a white to slightly yellowish powder, depending on the extent of deionization (See Process Flow Diagram Above). Pullulan products designated PI have undergone extensive deionization, and are white in color. Pullulan, which has been purified and designated as food grade may be white to slightly yellowish.

All products contain greater than 90% Pullulan, and less than 6% moisture. The residue on ignition is less than 1.5% for PF-20 and less than 5.0% for PF-10. The pH of all Pullulan products ranges from 5.0 to 7.0. The content of mono, di- and oligosaccharides (on a dry basis) is less than 10%. HPLC analysis of the saccharides in Pullulan have shown that greater than 33% are composed of two glucose molecules or less, and that 95.6% have a degree of polymerization (DP) less than

Section II Chemical Identity

10 [Hayashibara Internal data, 2000 Vol 2 Tab 13].

The viscosity of a 10% solution of Pullulan varies with the molecular weight of the product. PF-20, with a mean molecular weight of 200,000 has a viscosity ranging from 100-180 mm²/s. PF-10 has a mean molecular weight of 100,000, and its viscosity ranges from 15-25 mm²/s. Microbiological profiles show that the products are negative on a 25-gram basis for contaminants of public health significance, such as *Salmonella sp.*, *E. coli*, and *Staphylococcus aureus*.

3. Analysis of 10 Lots

In order to demonstrate that Hayashibara Company, Ltd. is able to consistently manufacture Pullulan to meet published specifications, the company has analyzed 10 lots of PF-20 produced over a period of time. The results are shown in Table 5 (below).

The data show that product purity ranged from 91.2 to 95.0%. Oligosaccharide content ranged from 5.0 to 7.2%, and moisture ranged from 2.2 to 3.1%. Residue on ignition ranged from 0.0 to 0.16%, and pH ranged from 5.53 to 6.02. The product had a mean viscosity of 150 mm²/s, and the metals content of all lots was less than the published specification. Interestingly, the data showed that the products contained no viable microorganisms.

4. Certificates of Analysis for Pullulan PF-10

Table 4 depicts the results of three Certificates of Analysis for Pullulan PF-10. Significantly, these analyses were carried out on Pullulan produced in three different years. Lot number 7B18 was produced February 18, 1997, lot number 8B18 was produced February 18, 1998, and lot number CB21 was produced March 1, 2001. The results showed that loss on drying was less than 6.0%, residue on ignition was less than 3.0%, and the pH ranged from 5.51 to 5.72. Two lots (7B18 and CB21) yielded the same color in aqueous solution, 0.052, and the third lot yielded a very similar value, 0.048. Protein content of the products ranged from 0.10 to 0.22%. Heavy metals and arsenic were less than

Section II Chemical Identity

5ppm and 2ppm, respectively. As in the data for PF-20 (shown in Table 5) no viable counts of microorganisms were detected for any of the lots.

5. Lead and Specific Heavy Metal Analyses

In order to ensure that HBC Pullulan products met the published specifications for lead, analyses were performed by an independent laboratory (Institut Européen de l'Environnement de Bordeaux, Bordeaux, France; IEEB) on 10 lots of Pullulan PI-20. The results are shown in Table 6. All lots contained less than 0.1ppm lead as analyzed by Atomic Absorption. The concentrations for lead ranged from 0.0ppm to 0.09ppm. The company also performed specific heavy metal analyses on three lots of HBC Pullulan PF-20, lot numbers: 1K01, 1J31, and 1J20 and two lots of HBC Pullulan PI-20, numbers: 1K06 and 11117. The lots were tested for cadmium, lead, mercury, and arsenic. The data, as shown in Table 7, indicate that cadmium, lead, and mercury were all below the level of detection, and that levels of arsenic were near the level of detection, ranging from 0.079 to 0.098 mg/kg for PF-20 and from 0.020 to 0.033 mg/kg for lots of PI-20. These data indicate that the concentration of lead in Hayashibara Pullulan is less than the recommended tolerance limits for GRAS substances with high consumption levels (<0.1ppm) contained in the Agency's advance notice of proposed rule making "*Lead in Food and Color Additives and GRAS Ingredients; Request for Data*" (*Federal Register*, February 4, 1994).

6. Mycotoxin Levels

Since the fungal species *Aureobasidium pullulans* is used to produce Pullulan, the Hayashibara Company, Ltd. had the IEEB measure the level of mycotoxins in three commercial lots of their Pullulan PF-20 product and in two commercial lots of PI-20. All mycotoxins tested were below the level of detection, as shown in Table 8 below. The assays were performed as an additional demonstration of safety. The Sponsor is not aware of any information, either from the literature or from in-house sources, that suggests that *A. pullulans* produces a mycotoxin(s).

Section II Chemical Identity

Table 4
Certificates of Analysis for Pullulan PF-10

| Lot No. | 7B18 | 8B18 | 1C21 |
|---|-------------|-------------|-------------|
| Loss on Drying (%) | 4.7 | 5.5 | 4.6 |
| Moisture (%) | 4.7 | - | - |
| Residue on Ignition (%) | 2.56 | 2.71 | 2.72 |
| Residue on Ignition (Sulfate) (%) | 3.33 | 3.52 | 3.54 |
| pH in Aqueous Solution | 5.62 | 5.51 | 5.72 |
| Color in Aqueous Solution | 0.048 | 0.052 | 0.052 |
| Viscosity (cP) | 19 | 18 | - |
| Viscosity (mm²/s) | 19 | 19 | 21 |
| Protein (%) | 0.10 | 0.20 | 0.22 |
| Heavy Metals as Pb (ppm) | <5 | <5 | <5 |
| Arsenic as As₂O₃ (ppm) | <2 | <2 | <2 |
| Standard Plate Count (CFU/g) | 0 | 0 | 0 |
| Coliform Organisms | Negative | Negative | Negative |
| Yeast and Mold | - | 0 | 0 |

0000510001

Section II Chemical Identity

Table 5: Analytical Test Results of 10 Lots of HBC Pullulan PF-20

| Item | Specifications | Lot No. | | | | | | | | | | Mean | Max. | Min. | |
|-------------------------------|-----------------------------------|---------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|------|------|---|
| | | 00308 | 105 | 00329 | 00202 | 00301 | 00126 | 00119 | 91027 | 91117 | 91124 | | | | |
| Appearance | Tasteless, odorless, white powder | Passed | Passed | Passed | Passed | Passed | Passed | Passed | Passed | Passed | Passed | Passed | - | - | - |
| Purity (dry basis) | >90% | 91.2 | 92.7 | 91.6 | 92.4 | 92.9 | 95.0 | 92.5 | 93.5 | 93.7 | 92.7 | 92.8 | 95.0 | 91.2 | |
| Oligosaccharides (dry basis) | <10% | 8.7 | 7.3 | 8.4 | 7.6 | 7.2 | 5.0 | 7.4 | 6.5 | 6.3 | 7.3 | 7.2 | 8.7 | 5.0 | |
| Moisture content | <6.0% | 2.4 | 2.2 | 2.5 | 2.9 | 2.5 | 2.8 | 2.5 | 2.6 | 2.7 | 3.1 | 2.6 | 3.1 | 2.2 | |
| Residue on ignition | <1.5% | 0.07 | 0.04 | 0.08 | 0.08 | 0.05 | 0.04 | 0.16 | 0.00 | 0.03 | 0.04 | 0.06 | 0.16 | 0.00 | |
| Viscosity (mm ² h) | 100-180 | 132 | 139 | 152 | 174 | 151 | 139 | 136 | 179 | 152 | 149 | 150 | 179 | 132 | |
| Lead | <1 ppm | Passed | Passed | Passed | Passed | Passed | Passed | Passed | Passed | Passed | Passed | Passed | - | - | - |
| Arsenic | <2 ppm | Passed | Passed | Passed | Passed | Passed | Passed | Passed | Passed | Passed | Passed | Passed | - | - | - |
| Heavy metals | <5 ppm | Passed | Passed | Passed | Passed | Passed | Passed | Passed | Passed | Passed | Passed | Passed | - | - | - |
| pH | 5.0-7.0 | 5.64 | 6.02 | 5.93 | 5.53 | 5.85 | 5.68 | 5.83 | 5.67 | 5.61 | 5.74 | 5.75 | 6.02 | 5.53 | |
| Coliform | <10/g | Neg. | Neg. | Neg. | Neg. | Neg. | Neg. | Neg. | Neg. | Neg. | Neg. | - | - | - | |
| Yeast | <100/g | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| Molds | <100/g | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| Salmonella | Neg/25g | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| E.coli | Neg/25g | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| S.aureus | Neg/25g | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |

000052

Section II Chemical Identity

Table 6
Analyses of Lead Levels in HBC Pullulan PI-20
(10 Lots)

| Lot No. | Lead (ppm) |
|---------|------------|
| 91027 | 0.02 |
| 91117 | 0.00 |
| 91124 | 0.00 |
| 00119 | 0.01 |
| 00126 | 0.00 |
| 00202 | 0.01 |
| 00301 | 0.00 |
| 00308 | 0.00 |
| 00329 | 0.00 |
| 00405 | 0.09 |

Table 7
Analyses of Specific Heavy Metals in HBC Pullulan PF-20 and PI-20
(5 Lots)

| Lot No. | Cadmium (mg/kg) | Lead (mg/kg) | Mercury (mg/kg) | Arsenic (mg/kg) |
|--------------|-----------------|--------------|-----------------|-----------------|
| PF-20 | | | | |
| 1J20 | <0.005 | <0.05 | <0.01 | 0.098 |
| 1J31 | <0.005 | <0.05 | <0.01 | 0.079 |
| 1K01 | <0.005 | <0.05 | <0.01 | 0.087 |
| PI-20 | | | | |
| 1K06 | <0.005 | <0.05 | <0.01 | 0.033 |
| 11117 | <0.005 | <0.05 | <0.01 | 0.020 |

Section II Chemical Identity

Table 8
Mycotoxin Levels of Hayashibara Pullulan

| Mycotoxin | Assay Method | Mycotoxin Concentration (µg/kg) Pullulan PF-20 Lot No. 1K01 | Mycotoxin Concentration (µg/kg) Pullulan PF-20 Lot No. 1J31 | Mycotoxin Concentration (µg/kg) Pullulan PF-20 Lot No. 1J20 | Mycotoxin Concentration (µg/kg) Pullulan PI-20 Lot No. 1K06 | Mycotoxin Concentration (µg/kg) Pullulan PI-20 Lot No. 11117 |
|-------------------|----------------------|--|--|--|--|---|
| Aflatoxin B1 | NF EN 12955 (Oct 99) | <2 | <2 | <2 | <2 | <2 |
| Aflatoxin B2 | NF EN 12955 (Oct 99) | <2 | <2 | <2 | <2 | <2 |
| Aflatoxin G1 | NF EN 12955 (Oct 99) | <2 | <2 | <2 | <2 | <2 |
| Aflatoxin G2 | NF EN 12955 (Oct 99) | <2 | <2 | <2 | <2 | <2 |
| Zearalenone | HPLC | <10 | <10 | <10 | <10 | <10 |
| Sterigmatocystine | HPLC | <50 | <50 | <50 | <50 | <50 |
| Ochratoxin | HPLC | <2 | <2 | <2 | <2 | <2 |

000054

Section II Chemical Identity

7. Antibiotic and Aureobasidin Production

The presence of antibiotics is not anticipated from the information available to the Sponsor; however, for additional assurance three lots of HBC Pullulan PF-20 and two lots of PI-20 were tested for antimicrobial activity. The assays were performed at the IEEB. This is a laboratory that performs official assays for the Ministry of Health, the Ministry of the Environment and other governmental agencies. This study was performed according to the U.N. Food and Agriculture Organization protocol using bacterial strains recommended for the antibiotic assay.

Using this test system, no antimicrobial activity was detected in the Pullulan products. Table 9 displays the results.

Strains of *Aureobasidium pullulans* have been shown to produce a group of antifungal agents termed Aureobasidins (Takesako, K, *et al.*, J Antibiotics 44: 919-24, 1991). These are cyclic depsipeptides formed by eight L- α -amino acids. Three or four of the amino acids are N-methylated, and a hydroxy acid binds to both ends to form a ring structure (Ikai, K, *et al.*, J Antibiotics 44: 925-33, 1991). Aureobasidins are not structurally similar to known mycotoxins [Kimoto, *et al.*, 1997 Vol 3 Tab 22]. More importantly, Aureobasidins have been shown to be non-toxic in mice (Takesako, K, *et al.*, J Antibiotics 46: 1414 -1420, 1993). Aureobasidin was administered in a single dose at concentrations up to 50mg/ml to 5 female mice. Treatments were given intravenously, intraperitoneally, subcutaneously and per os, and the mice were observed for 7 days. LD₅₀ values were 231, approximately 1,000, >1,000, and >1,000mg/kg when given by the respective routes of administration. Further, data from studies on animals and humans, and commercial consumption of Pullulan in Japan over a period of more than 20 years has not indicated any safety associated concern associated with the production strain.

Section II Chemical Identity

Table 9: Antibacterial Activities in HBC Pullulan Products

| Bacteria | ATCC ^a number | Antibacterial activities in HBC Pullulan PF-20 and PI-20 ($\mu\text{g/g}$) ^b | | | | |
|------------------------------|-----------------------------|--|----------------------|----------------------|----------------------|-----------------------|
| | | lot n° 1K01 PF-20 | lot n° 1J20 PF-20 | lot n° 1J20 PF-20 | lot n° 1K06 PI-20 | lot n° 11117 PI-20 |
| <i>Bacillus subtilis</i> | 6633 | negative | negative | negative | negative | negative |
| <i>Bacillus cereus</i> | 9634 | negative | negative | negative | negative | negative |
| <i>Enterococcus faecalis</i> | 8043 | negative | negative | negative | negative | negative |
| <i>Escherichia coli</i> | 8789 | negative | negative | negative | negative | negative |
| <i>Serratia marcescens</i> | 14756 | negative | negative | negative | negative | negative |
| <i>Staphylococcus aureus</i> | 6538 | negative | negative | negative | negative | negative |

a: American Type Culture Collection. b: μg of antibiotic per gram sample.

000056

Section II Chemical Identity**8. Microbial Content of Commercial HBC Pullulan Products**

Three lots of HBC Pullulan PF-20 and two lots of PI-20 were tested for microbial contamination. The assays were performed at the IEEB. This study was performed using methods recognized by the U.N. Food and Agriculture Organization and the European Union. The results are presented in Table 10.

The results indicated that HBC Pullulan was very nearly free of microbial cells. The product was below the level of detection for all microbial pathogens, and contained less than 100 total mesophilic organisms. A small number of flat sour spores (<7) were detected in each of the three lots of PF-20 tested and in one lot of PI-20.

Section II Chemical Identity

Table 10
Microbial Analyses of Commercial HBC Pullulan Products

| Microbial Variable | Method | Pullulan PF-20 Lot No. 1K01 CFU/g | Pullulan PF-20 Lot No. 1J20 CFU/g | Pullulan PF-20 Lot No. 1J31 CFU/g | Pullulan PI-20 Lot No. 1K06 CFU/g | Pullulan PI-20 Lot No. 111117 CFU/g |
|---|-----------------|---|---|---|---|---|
| Total Mesophilic Bacteria | NF V 08-051 | <100 | <100 | <100 | <100 | <100 |
| Coliforms | NF V 08-050 | <1 | <1 | <1 | <1 | <1 |
| Fecal Coliforms | NF V 08-060 | <1 | <1 | <1 | <1 | <1 |
| <i>Staphylococcus sp.</i> Coagulase Positive | NF V 08-057-1 | <1 | <1 | <1 | <1 | <1 |
| <i>Salmonella sp.</i> /25 g | NF V 08-052 | Negative | Negative | Negative | Negative | Negative |
| Anaerobic Sulphite Reducers @ 46°C | XP V 08-061 | <1 | <1 | <1 | <1 | <1 |
| <i>Clostridium perfringens</i> | NF V 08-056 | <1 | <1 | <1 | <1 | <1 |
| <i>Listeria monocytogenes</i> /10 g | NF V 08-055 | Negative | Negative | Negative | Negative | Negative |
| <i>Shigella sp.</i> /25 g | Internal Method | Negative | Negative | Negative | Negative | Negative |
| <i>Campylobacter sp.</i> /25 g | NF ISO 10272 | Negative | Negative | Negative | Negative | Negative |
| Flat sour spores | Gerber US | 3 | 7 | 6 | <1 | 2 |
| Thermoresistant spores | NF V 08-407 | <3 | <3 | <3 | <3 | <3 |

000058

Section II Chemical Identity**M. Test Methods**

Hayashibara Company, Ltd. can provide written test methods for each of the chemical and microbiological variables in its specifications for Pullulan products. These methods are approved in Japan and correspond to either AOAC Methods of Analysis or the methods provided by FDA in the *Bacterial Analytical Manual* (BAM).

N. Stability of Pullulan

The storage stability of 5 lots of Hayashibara Pullulan was assayed in its commercial packaging materials. The cartons were stored in the Product Storage Room at room temperature (10-30°C: RH 40-65%). Samples were tested periodically over a 24-month storage period for moisture, pH, viscosity, fungi, Pullulan purity, and oligosaccharide content. The analytical results were reported after the first month, then every 3 months for the first 12 months, and then every 6 months for the final 12 months of the test period.

The stability study showed that virtually no change had occurred in the test criteria throughout the test period, and that the product remained within the prescribed specifications (with one exception for moisture at 24 months for Lot No. 50303). Based on the test results, it was concluded that HBC Pullulan was stable for 24 months of storage under the test conditions used. Data is provided in Appendix 5.

O. Explanation of the Lot Code

HBC Pullulan is assigned lot numbers based on two coding systems. One code uses all numbers and the second one is alpha-numeric. The former code is used to identify Pullulan lots that are sold to one specific customer. For various reasons Hayashibara is interested in being these able to easily identify these lots. An examples of the lots that use only

Section II Chemical Identity

able to easily identify these lots. An examples of the lots that use only numbers would be "10227". The first digit "1" indicates the year, in this example the year is 2001. The second digit, "02", signifies the month, in this case, February, and the last two digits, "27", denote the day. Thus, Lot No " 10227" was manufactured on February 27, 2001. In the second coding system the only change is that the second and third digits, representing the month are replaced by a letter. For instance, "A" would be January, "B" February and so on. For the example above, the code would be "1B27".

P. Summary

Pullulan is a polysaccharide elaborated extracellularly by *Aureobasidium pullulans* in nature. The organism is ubiquitous. Hayashibara Pullulan is commercially produced by a non-pathogenic and non-toxigenic strain of *A. pullulans* utilizing corn syrup as the substrate. Pullulan has a linear structure comprised of maltotrioses in which three glucose units are linked through α - 1,4-glucosidic bonds. The maltotrioses are in turn linked to a series of other maltotrioses through α -1,6-glucosidic bonds creating a long stair-step-type structure. The molecular weight of Pullulan can range from 50,000 up to several million daltons. Pullulan is very similar in composition to the amylopectin fraction of typical food starches, found in corn and wheat. However, due to its size and bond configuration, it acts as a dietary fiber in the human body.

Commercial Pullulan is produced under current Good Manufacturing Practice, which results in product purity comparable to that specified for dextrin in the Food Chemicals Codex, 4th Edition. The products have been extensively tested for the presence of antibiotics, mycotoxins, heavy metals, and pathogens. No contaminants of public health significance have been observed. The Food Chemical Section, Environmental Health Department, Ministry of Health and Welfare approved Pullulan as a food ingredient in Japan. It is also listed in the Standards for Ingredients of Drugs and is widely used as a pharmaceutical additive for bulking and stabilization of tablets in Japan.

Section III

000061

Section III. Technical Effects, Intended Uses, Consumption Estimates, and Information on Self-Limiting Levels

Section III. **Intended Uses, Functional Effects, Consumption Estimates, and Self-Limiting Levels**

Pullulan has been used in Japan for more than 20 years as both an indirect food ingredient for coatings on food packaging, and as a direct food additive in a variety of applications. This experience provides examples upon which to base physical and technical functional effects, the intended uses, consumption estimates and self-limiting aspects of this product.

A. Intended Uses, Functional Effects, and Use Levels

Hayashibara Pullulan has a variety of potential uses in the U.S. These potential products are based on the experience of use in Japan as well as Hayashibara company research and information from U.S. patents. This information provides realistic intended uses, functional effects and use levels that are associated with the use of Pullulan under current Good Manufacturing Practice.

1. Intended Uses

The first column of Table 11 includes several specific types of products (Intended Uses). The second column lists the 43 FDA food categories found at 21 CFR §170.3(n) that are correlated to the food products in the first column. These are provided to show the variety of food categories in which Pullulan may be used.

The intended uses of Pullulan fall within three general categories. They include use as an ingredient directly added to foods, as a film, and as an excipient. The following is a brief description of each use.

Food Ingredient--One primary category of intended use of Pullulan is as a multifunctional food ingredient that provides physical and technical effects that improve the quality of a variety of food products. Several of the Japanese food products provided in Table 11 would be likely candidates for use in the US. It is proposed that the use levels in Japan provide a reasonable guide for the use levels in the US. An additional intended use in this category is as a fiber source in food products.

Section III. Technical Effects, Intended Uses, Consumption Estimates, and Information on Self-Limiting Levels

Film--A second category of intended use for Pullulan is for the production of edible films. Pullulan can be used to produce an edible film that has been sold in Japan as a fast dissolving breath "mint". The film can also be formed into soft or hard capsules for nutrient delivery. Pullulan has been shown to be an acceptable alternative to gelatin. It can be used as essentially the only material in a capsular matrix, or it may be combined with other materials [US Patent 5,411,945 Vol 4 Tab 48; US Patent 4,623,394, Vol 4 Tab 47]. Food products can be dipped or sprayed with Pullulan, resulting in a film coating that provides esthetic enhancement, a matrix to hold flavors, and an oxygen barrier to preserve flavor, color and protect quality.

Excipient--Pullulan can be used in several excipient applications. These would include: a) Coating to slow tablet deterioration, increase shock resistance, reduce cracking, reduce color deterioration, and improve finish and gloss, b) Granulation to add stability and reduce particle size, c) Pelleting to increase stability, increase binding strength, and prevent elution, d) Binding agent for tablets, and e) Direct blending with nutrient substances for tableting to provide timed release of ingredients [Ohta, *et al.*, 1985a Vol 3 Tab 30 and Ohta, *et al.*, 1985b Vol 3 Tab 31]. Depending on the type of excipient application, the amount of Pullulan as a percentage of total tablet weight would range from 1.66% (for coatings) to more than 95% (for binding).

2. Physical and Technical Functional Effects and Use Levels

Pullulan has several physical and technical functional effects that suggest its use for a number of products. The various functional effects listed in Table 11 can be correlated to the list of 32 physical or technical functional effects published by the FDA for which direct food ingredients may be added to food. These are codified at 21 CFR §170.3 (o) (1-32). The following is a list of the appropriate classifications of physical and technical functional effects under 21 CFR §170.3 (o).

Section III. Technical Effects, Intended Uses, Consumption Estimates, and Information on Self-Limiting Levels

(8) "Emulsifiers and emulsifier salts": Substances, which modify surface tension in the component phase of an emulsion to establish a uniform dispersion or emulsion.

(14) "Formulation aides": Substances used to promote or produce a desired physical state or texture in food, including carriers, binders, fillers, plasticizers, film-formers, and tableting aids, etc.

(16) "Humectants": Hygroscopic substances included in food to promote retention of moisture, including moisture-retention agents and antidusting agents.

(20) "Nutrient supplements": Substances that are necessary for the body's nutritional and metabolic processes.

(24) "Processing aids": Substances used as manufacturing aids to enhance the appeal or utility of a food or food component, including clarifying agents, clouding agents, catalysts, flocculants, filter aids, and crystallization inhibitors, etc.

(28) "Stabilizers and thickeners": Substances used to produce viscous solutions or dispersions, to impart body, improve consistency, or stabilize emulsions, including suspending and bodying agents, setting agents, jellying agents, and bulking agents, etc.

(29) "Surface-active agents": Substances used to modify surface properties of liquid food components for a variety of effects, other than emulsifiers, but including solubilizing agents, dispersants, detergents, wetting agents, rehydration enhancers, whipping agents, foaming agents, and defoaming agents, etc.

(31) "Synergists": Substances used to act or react with another food ingredient to produce a total effect different or greater than the sum of the effects produced by the individual ingredients.

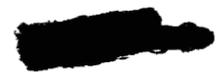
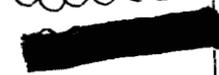
(32) "Texturizers": Substances, which affect the appearance or feel of the food.

Section III. Technical Effects, Intended Uses, Consumption Estimates, and Information on Self-Limiting Levels

TABLE 11 . Intended Use and Functional Effects

| Intended Use | FDA Food Category¹ | Use Level (%) | Physical or Technical Functional Effect² |
|---|--|----------------------|--|
| Confectionery (cookies, doughnuts, wafers etc.) | Baked Goods, Baking Mixes | 0.93-3.0 | Provides viscosity and acts as a binder |
| Artificial rice and noodles | Grain Products and Pastas | 0.4 | Acts as a binder |
| Flour for tempura | Grain Products and Pastas | 1.0 | Provides viscosity and adhesiveness |
| Baked ground fish-meat products | Fish Products | 0.2 | Improves quality and shelf life |
| Ham and processed meats | Meat Products | 0.2 | Acts as a binder and retains moisture |
| Glaze for meat items | Gravies and Sauces | 0.2-1.04 | Acts as a binder and carrier for flavors |
| Dried fish-meat, snack type processed meat products | Fish Products | 1.0-3.0 | Oxygen barrier and/or imparts gloss |
| Processed Marine Products (Processed Sea Weed, Dried Seafood) | Processed Vegetables and Vegetable Juices | 1.0-3.0 | Oxygen barrier and/or imparts gloss |
| Instant chow mein | Processed Vegetables and Vegetable Juices | 0.5-3.0 | Oxygen barrier, and improves quality and shelf life |
| Dried pork and vegetables for instant chow mein | Processed Vegetables and Vegetable Juices; Meat Products | 3.0 | Packing material to prevent oxidation and product scattering, edible packaging |
| Nuts | Nuts and Nut Products | 0.5-3.0 | Oxygen barrier, and improves quality and shelf life |
| Frozen Food Products | Processed Vegetables and Vegetable Products; Processed Fruits and Fruit Juices | 0.4 | Oxygen barrier, maintains quality |
| Fruits | Fresh Fruits and Fruit Juices | 1.5-3.0 | Prevents oxidation and maintains quality |
| Canned tangerines | Processed Fruits and Fruit Juices | 0.6 | Prevents turbidity |
| Pickled Foods | Processed Vegetables and Vegetable Products | 3.0 | Improves texture, maintains quality |
| Tofu and Miso | Plant Protein Products | 0.5-0.8 | Improves quality |

000065



Section III. Technical Effects, Intended Uses, Consumption Estimates, and Information on Self-Limiting Levels

TABLE 11 . Intended Use and Functional Effects (Continued)

| Intended Use | FDA Food Category ¹ | Use Level (%) | Physical or Technical Functional Effect ² |
|---|---|---------------|---|
| Processed eggs | Egg Products | 0.4 | Maintains quality |
| Soup solids | Soups and Soup Mixes | 0.3-0.4 | Water soluble edible ingredient and provides viscosity |
| Condiments, Seasoning (Mayonnaise, Seasoned Powder) | Condiments and Relishes | 3.0-5.0 | Viscosifier, |
| Soy sauce, other sauces and gravies | Gravies and Sauces | 0.3-3.0 | Provides viscosity |
| Japanese Confectionery (Rice and Bean Sweets, Rice Cakes and Crackers, Sugar Coated Sweets) | Confections and Frostings | 0.4-5.0 | Viscosifier, coating agent, binder |
| Western Confectionery (Candies, Tablet Candies, Chewing Candies, Snacks) | Confections and Frostings | 1.0-5.0 | Binder, excipient, texturizer, imparts gloss, plasticizer, |
| Milk Based Desserts (Ice Cream, Whipped Cream) | Milk Products | 0.4-0.9 | Foam enhancer |
| Sweet Syrups | Sweet Sauces, Toppings, and Syrups | 0.6 | Viscosifier, binder, adjunct for flavorings and colors |
| Chewing Gum | Chewing Gum | 0.2-0.5 | Texturizer, prevents brittleness |
| Black and Japanese tea | Coffee and Tea | 0.3-0.4 | Water-soluble Pullulan-laminated tea bag, oxygen barrier, maintains quality |
| Tablets, Coated | Confections and Frostings | 1.66 | Coating agent, excipient |
| Additional Uses Anticipated in the US Market | | | |
| Various Non-Alcoholic Beverages | Beverages and Beverage Bases, Non-Alcoholic | 0.3 | Viscosifier |
| Breakfast Cereals | Breakfast Cereals | 0.5 | Coating agent, texturizer |
| Cheeses | Cheeses | 0.7 | Binder, stabilizer |
| Salad Dressings | Fats and Oils | 0.25 | Viscosifier, stabilizer |
| Commercial Jams and Jellies | Jams and Jellies, Commercial | 0.5 | Thickener |
| Nutritional Bars | Confections and Frostings | 10.0 | Binder, source of dietary fiber |
| Capsules | - | 15.0-90.0 | Film-former |

¹From 21 CFR § 170.3(n), 32 physical and technical effects. ²Can be correlated with FDA physical and technical functional effects from 21 CFR § 170.3(o).

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Section III. Technical Effects, Intended Uses, Consumption Estimates, and Information on Self-Limiting Levels

B. Consumption and Exposure Estimate

1. Consumption

Pullulan is a polysaccharide composed exclusively of glucose molecules, as are other common food grade ingredients such as starch, dextrin and maltodextrin. All four substances consist of glucose units linked through α -1,4-glucosidic and α -1,6-glucosidic bonds. Dextrin and maltodextrin have been affirmed GRAS under 21 CFR Part 184. The dextrans and Pullulan are particularly similar to the amylopectin fraction of food-grade starches, which has always been a significant part of the human diet. Pullulan is fundamentally resistant to degradation by human digestive enzymes; whereas, starches, dextrin and maltodextrin are usually digestible. Modifications in processing can result in non-digestible products. If these are not digestible, they are commonly enzymatically hydrolyzed by the bacterial flora in the large intestine.

Since the introduction of Hayashibara Pullulan in 1976, millions of kilograms have been sold into the Japanese food market. As of this date, the company is unaware of any consumer complaint resulting from the consumption of Pullulan. Unlike in the US, where many food-associated adverse events go unreported, the Japanese consumer is well-known for either directly contacting the food processor, or making use of one of the many regulatory avenues available for registering a complaint. Therefore, it is likely that Hayashibara would have been notified had any adverse effect been reported.

2. Exposure Estimate

In order to estimate the probable human exposure to HBC Pullulan on a continual daily basis, Hayashibara Company, Ltd. has relied upon examples of commercial formulations from Japan, examples of potential Pullulan use from the recent patent literature, and the results of its own food technology research. Exposure to Pullulan was estimated in

Section III. Technical Effects, Intended Uses, Consumption Estimates, and Information on Self-Limiting Levels

accordance with the agency's guidance document, *Estimating Exposure to Direct Food Additives and Chemical Contaminants in the Diet* [FDA, September, 1995 Vol 2 Tab 9].

Use of Pullulan was estimated using the 94 sub-categories included in the document from the National Academy of Sciences titled "GRAS Food Additive Categories and Sub-Categories" (data not shown). Since this document does not correlate these 94 food sub-categories with consumption, the food categories were combined so that they were consistent with the food categories from USDA's Continuing Survey of Food Intakes by Individuals (CSFII) [Enns, *et al.*, 1997 Vol 2 Tab 8]. The CSFII food categories, along with percent Pullulan use, mean daily food intake, and subsequent Pullulan intake in grams per day are provided in Table 12. This Table presents mean intake data for adults 20 years and over (eaters only).

For example, three food sub-categories (white bread, dark bread, rolls) from the National Academy of Science list were averaged to yield the average use level for Yeast Bread and Rolls shown on Table 12 (the CSFII categories). Since the CSFII intake data is statistically valid, the percentage use level for Pullulan may be multiplied by the mean intake indicated in the CSFII database to yield an estimated exposure to Pullulan from a given food category (Pullulan use % x Daily mean food intake per food category = Daily mean Pullulan intake per food category). The resulting totals for each food category were then summed to yield a total mean daily intake.

The total mean daily intake of Pullulan used as a food ingredient is calculated to be 9.4 grams. This amount was doubled to 18.8 grams, to provide an estimate of the 90th percentile, in accordance with the guidance document, *Estimating Exposure to Direct Food Additives and Chemical Contaminants in the Diet* [FDA, September, 1995 Vol 2 Tab 9].

In addition to the estimated intake for the items listed in the CSFII categories, there are three specialized categories in Table 12 listed below

Section III. Technical Effects, Intended Uses, Consumption Estimates, and Information on Self-Limiting Levels

the calculated total mean daily consumption. In conversations with the Agency it was suggested that these should be listed as separate line items outside the normal daily consumption calculation because the products would be relatively restricted to specific populations. The three categories are the use of Pullulan for producing capsules, its use as an excipient in tablets, and its use in a dietary supplement as a source of fiber (no health claim is inferred).

The daily intake of Pullulan as a capsule would be approximately 0.69g per day. Industry figures show that the average consumption of capsules in the US is 0.71 per day and average weight of a capsule is 0.97g [Proprietary Report to Hayashibara International Inc., 2001 Vol 3, Tab 36]. The International Pharmaceutical Excipient Council (IPEC) estimates that the average adult in the US consumes 5 tablets per day, and that the average tablet weighs 0.5 grams [IPEC, Personal Communication, 2001 Vol 2 Tab 16]. Therefore, Pullulan consumption as an excipient would range from 0.415 grams/day, if it were used as a coating (1.66%) to 2.375 grams/day, if it were used as a binder ($\geq 95\%$). The daily intake of Pullulan as a fiber supplement is given as 1.5 to 15 grams/day, which is consistent with other soluble fiber supplements [Institute of Food Technologists Report, 1999 Vol 2 Tab 14].

Table 12. Estimated Intake of Pullulan in Selected Food Categories

| Based on the <i>Continuing Survey of Food Intakes by Individuals (1996)</i> | | | | |
|---|----------------------------------|------------------------|-----------------|---------------------|
| Major Food Codes | Food Category | Pullulan Use Level (%) | Food Intake (g) | Pullulan Intake (g) |
| | Baked goods, baking mixes | | | |
| 51 | Yeast Breads and Rolls | 0.5 | 50 | 0.25 |
| 52, 55 | Quick breads, pancakes, etc. | 0.5 | 20 | 0.1 |
| 53 | *Cakes, cookies, pastries, pies | 3 | 38 | 1.14 |
| 58 | Mixtures mainly grain | 0 | 107 | 0 |
| | Breakfast cereals | | | |
| 571 - 574, 578 | Ready-to-eat cereals | 0.5 | 17 | 0.085 |
| | Grain products and pastas | | | |
| 562 | Rice | 0.4 | 19 | 0.076 |
| 561 | Pasta | 0.4 | 21 | 0.084 |
| 54 | Snack foods(crackers, chips) | 10 | 12 | 1.2 |
| | Total Vegetables | | 132 | |
| | Fresh vegetables | 0 | 0 | 0 |

Section III. Technical Effects, Intended Uses, Consumption Estimates, and Information on Self-Limiting Levels

| | | | | |
|--|---|------|------|--------------------|
| | Processed vegetables, juices | 0.4 | 74 | 0.296 |
| | Other Vegetables | 3 | 46 | 1.38 |
| | Total Fruits | | 162 | |
| 612 | Citrus juices | 1 | 59 | 0.59 |
| 621 | Dried fruits | 0 | 1 | 0 |
| Table 12 Estimated Intake of Pullulan in Selected Food Categories (Continued) | | | | |
| 641,642 | Non-citrus juices and nectars | 1 | 26 | 0.26 |
| | Fruits and mixtures | 0 | 0 | 0 |
| | Milk products | | | |
| 114 | Yogurt | 4 | 8 | 0.32 |
| 131,132, 133, 134 | Milk desserts | 0.4 | 24 | 0.096 |
| 140-147 | Cheese | 0.7 | 16 | 0.112 |
| 116, 118 | Milk-Based Beverages | 0.4 | 34 | 0.136 |
| | Meat Products | | | |
| 252 | Sausages, processed meats | 0.2 | 21 | 0.42 |
| | Pork** | 0.2 | 5** | 0.1 |
| | Eggs | | | |
| 321,323,324, 33,34 | Egg Products*** | 0.4 | 9*** | 0.045 |
| | Legumes | | | |
| | Legumes | 0.9 | 28 | 0.252 |
| | Nuts and Nut Products | | | |
| | Nuts and Nut Products | 0 | 0 | 0 |
| | Fats and Oils | | | |
| 832 | Salad dressings | 0.25 | 8 | 0.02 |
| | Total sugars and sweets | | | |
| 917,918 | Candy (Soft and Hard) | 2.72 | 7 | 0.19 |
| 911 - 916 | Other Sugar Products**** | 3.16 | 15 | 0.474 |
| | Beverages Non-Alcoholic | | | |
| | Coffee | 0.3 | 254 | 0.762 |
| 923 | Teas | 0.4 | 128 | 0.512 |
| 925 | Low Cal. Fruit drinks and aides | 0.2 | 18 | 0.36 |
| 92400100 | Low Cal. Carbonated drinks | 0.2 | 74 | 0.148 |
| | Total Mean Intake (grams) | | | 9.408 |
| | 90th Percentage Mean Intake | | | 18.816 |
| | Dietary Supplement | | | 1.5-15 |
| | Average as capsule material (grams) | | | 0.069 |
| | Range as tablet excipient (grams) | | | 0.415-2.375 |
| | * Includes dietetic bars | | | |
| | ** Includes 50% of pork intake for prepared and smoked products | | | |
| | *** Includes 50% of egg intake for processed eggs | | | |
| | ****Includes Jams, Jellies, etc. | | | |

Note that zeros were entered for some food categories where significant Pullulan use is thought to be unlikely. These categories include: Baked good,

Section III. Technical Effects, Intended Uses, Consumption Estimates, and Information on Self-Limiting Levels

mixtures, mainly grain; Total vegetables, fresh vegetables; Total fruits, dried fruits; Total fruits, fruits and mixtures; and Nuts and nut products.

C. Self-Limiting Levels

The Sponsor asserts that the use of Pullulan will be self-limiting on the basis of the nature of the product itself. Pullulan meets the definition of a dietary fiber and could be consumed as such; however, it also has physical and technical functional effects when added to various food products. All fibers are self-limiting in foods, in that they are known to interact with other food ingredients in such a way that the total food system may be rendered unpalatable or unacceptable if the fiber is incorporated in amounts that negatively affect certain quality standards. Dr. George C. Fahey, Jr. of the University of Illinois, an expert in the area of the physiological effects of dietary fiber consumption, offered the following Opinion regarding the self-limiting nature of fiber ingredients, including Pullulan.

"The issue is occasionally raised as to whether one can consume too much fiber. Organoleptic properties of the fiber itself generally will prevent its over-consumption by humans. By themselves, many are dry, dusty, or gritty, and it is often a challenge of the food preparation specialist to mask their properties, when included in complete foods. Thus, maximum levels of incorporation of fibers into many different types of food products, including enteral formulas, have been defined. These levels are nearly always below the 35 grams/day quantity recommended by the various medical agencies" [Fahey, 2001 Expert Opinion, Appendix 1].

D. Other Limiting Factors

It is believed by the Sponsor that the estimates of both the percentage use of Pullulan and daily mean consumption of foods listed in the various categories are reasonable numbers; however, the calculated value of the total mean intake (grams) of Pullulan is believed to be higher than what would actually occur. There are additional limiting factors that would likely result in a lower consumption than presented.

Section III. Technical Effects, Intended Uses, Consumption Estimates, and Information on Self-Limiting Levels

1. The calculation assumes that every product in all categories will use Pullulan. Each category represents dozens if not hundreds of specific products. It is highly unlikely that Pullulan would provide benefit for more than a small fraction of the potential products.
2. The calculation assumes that there will be 100% market penetration in each of the categories and for each specific product (1. above). There are many different ingredients and additives that will compete for the functional effects provided by Pullulan. It is not likely that Pullulan will provide the most effective functional effect and subsequently the best quality final product in all the foods in which Pullulan could theoretically be used.
3. Pullulan is relatively more expensive than other ingredients or additives for which it might be substituted. The cost of Pullulan in Japan where the ingredient has been sold for approximately 20 years is approximately \$25(USD)/kg. While the cost benefit ratio may be sufficient to support this price in some specialized uses, it may not be cost effective for use for others since the food industry is one of the most cost sensitive sectors of the economy.

Section IV

000073

Section IV Safety

Section IV: HBC Pullulan:
Safety when Consumed as an Ingredient in Processed Foods

Introduction

This section contains a comprehensive discussion of available scientific data, and information that the Notifier has relied upon to reach its conclusion that HBC Pullulan is safe when used under current Good Manufacturing Practice (cGMP) as a direct additive to food. The basis upon which this determination of safety was made includes the similarity of Pullulan to other polyglucoses, the use of food grade materials, purity of the final product, the use of cGMP, safety of the source organism, acute and chronic studies on animals, digestibility in human and animals, and a history of safe use in humans in Japan. Citations to the published scientific literature that were included in the GRAS Report reviewed by an Expert Panel appear in brackets. A complete listing of each citation can be found in Appendix 2. Citations not included in the GRAS Report are given in parentheses in standard format.

A. Pullulan and Polyglucoses

Pullulan is a natural water-soluble polysaccharide elaborated extracellularly by *Aureobasidium pullulans*. It is commercially produced by a non-pathogenic, non-toxigenic strain of *A. pullulans* grown in media containing starch-based sugars and other food grade components. Pullulan has a linear structure comprised of maltotrioses, in which three glucose units are linked through α -1,4-glucosidic bonds. The maltotrioses are in turn linked to a series of other maltotrioses through α -1,6-glucosidic bonds, creating a long stair-step-type structure [Catley, 1986 Vol 2 Tab 5].

Pullulan, like several other edible molecules, are composed of only glucose molecules, which are bound together by α -1,4- and α -1,6-glucosidic bonds. Other common food-related substances that contain only glucose and the same α -1,4- and α -1,6-glucosidic bonds are dextrans, maltodextrins, and amylopectins. These three groups

Section IV Safety

have been considered or affirmed as GRAS (21 CFR 184.1277 & 184.1444). Polyglucose molecules found in common foodstuffs have been consumed for thousand of years, and investigators estimate that approximately 150 grams of these types of compounds are consumed daily in the diet [Southgate, 1998 Vol 4 Tab 40].

Differences between these molecules include the relative percentages of α -1,4 and α -1,6 bonds, and the tertiary structure. As mentioned, Pullulan has a stair-step structure, while amylopectins (a major component of starch) are bottlebrush in appearance. Dextrins and maltodextrins contain a variety of fragments broken down from amylopectin and amylose. Analyses of Pullulan, cornstarch and maltodextrin have shown that the percentage of α -1,6 bonds are 30, 20 and 5%, respectively [Whistler, *et al.*, 1984 Vol 4 Tab 53].

Another difference is the digestibility of these polyglucoses. Amylopectins, dextrins and maltodextrins are usually hydrolyzed to glucose by the human digestive enzymes; whereas, Pullulan is digested by bacteria in the large intestine. Conversely, starches, dextrins and maltodextrins can be modified by heat, chemical or enzymatic treatment. Some of these products, like Pullulan, are "resistant" to gastrointestinal tract digestion, and are hydrolyzed by bacterial enzymes in the colon. These modified resistant products have also been eaten in large quantities over the last several years. The final disposition of Pullulan and the resistant polyglucose molecules is to be converted into short-chain fatty acids that are thought to be of benefit to human body (Flickinger EA, *et al.* Journal of Nutrition 130:1267-73, 2000). It is thought that approximately 7% of all the energy used by humans is obtained from microbial metabolism in the large intestine (Cummings JH, *et al.* Journal of Parenteral and Enteral Nutrition 21(6):357-65, 1997).

Neither the Sponsor nor the Expert Panel for the GRAS review found any literature to suggest that the Pullulan molecule, consisting of glucose and α -1,4 and α -1,6 glycosidic bonds, would intrinsically present a greater safety concern than other polyglucose molecules.

Section IV Safety

While the lack of negative data is not proof of safety, it does provide a long history of the safe consumption of products with similar chemical structures. Hayashibara contacted Dr. George Fahey, Jr., who is a member of the National Academy of Science, Institute of Medicine's Panel on the Definition of Dietary Fiber. The Sponsor asked Dr. Fahey to give his expert opinion on the safety of glucose polymers as related to Pullulan. The full response is included in Appendix 1. A concluding statement is as follows, "Clinical studies conducted in our laboratory and in the laboratories of others have shown that a range of glucose polymers have been well tolerated by animals and humans, and no adverse health effects have been noted. Pullulan also is a glucose polymer and its linear structure with limited branching appears to reduce its digestibility. There is no indication that pullulan would be less well tolerated than another glucose-based oligosaccharide or polysaccharide mentioned above."

B. Commercial Pullulan

The commercial Pullulan product is produced with a purity and quality comparable to that specified for dextrin [Food Chemicals Codex, 4th Edition, 1996 Vol 2 Tab 10]. The safety of Hayashibara Pullulan for use in foods in general is supported by the fact that Pullulan meets a set of food grade specifications, is free of contaminants, and is free of the producing organism, *Aureobasidium pullulans*. The source organism itself is non-pathogenic and non-toxigenic, and Pullulan is manufactured under current Good Manufacturing Practice.

Pullulan has been approved as a food ingredient in Japan and safely used for more than 20 years as both an indirect food additive for coatings on food packaging and as a direct additive for a variety of applications. These applications were discussed in Section III above [Tsujiyaka, *et al.*, 1993 Vol 4 Tab 45].

The Food Chemical Section, Environmental Health Department, Ministry of Health and Welfare approved Pullulan as a food ingredient in Japan. It is also listed in the Standards for Ingredients of Drugs

Section IV Safety

and is used as a pharmaceutical additive for the bulking and stabilization of tablets in Japan [Ministry of Health and Welfare Opinion, 1986 Vol 4 Tab 41].

C. Pathogenicity of the Source Organism, *A. pullulans*

A. pullulans, the organism which elaborates Pullulan is ubiquitous in nature, and has generally been regarded as non-pathogenic and non-toxigenic [Wallenfels *et al.*, 1965 Vol 4 Tab 51]. There have been reports of its presence in clinical samples from isolated individuals. This has led to questions about possible pathogenicity [Salkin *et al.*, 1986 Vol 3 Tab 37, Kaxzrnarski *et al.*, 1986 Vol 3 Tab 21, and Giaradi *et al.* 1993 Vol 2 Tab 11]. However, as noted by Ajello, the mere isolation of a fungus from a lesion or from a clinical sample does not, *per se*, establish the isolate as a pathogen, especially when the organism is ubiquitous [Ajello, 1978 Vol 2 Tab 1]. Similarly, growth in immunosuppressed individuals does not indicate that it is a pathogen capable of establishing an infection in otherwise healthy individuals [Pariza, *et al.*, 2001 Vol 3 Tab 35].

For comparison, it should be noted that *Saccharomyces cerevisiae* (Brewer's or Baker's yeast), a harmless industrial yeast, has been implicated in several infections, but only in immunosuppressed patients [Sobels *et al.*, 1993, Vol 3 Tab 39, Tollermar *et al.*, 1992, Vol 4 Tab 44, and Tawfik, 1989 Vol 4 Tab 43]. Because there were a few reports suggesting a possible link between *Aureobasidium pullulans* and infection, Hayashibara International Inc. commissioned Dr. Donald G. Ahearn, and Dr. Libero Ajello, to provide their expert opinion concerning this issue. These two individuals are noted experts in the field of pathogenic mycology. Their written opinion is included in Appendix 1.

In brief, Drs. Ahearn, and Ajello, noted that *Aureobasidium pullulans* is a common black saprophobic mold, which is virtually ubiquitous in nature and in indoor environments. This mold is inhaled and ingested with fruits and vegetables everyday. These experts

Section IV Safety

indicated that early clinical studies failed to establish *Aureobasidium pullulans* as a pathogen. Additionally, reports of the involvement of this organism in clinical infections have been shown to be the result of misidentification of the isolated organism. Based on their years of experience, the researchers concluded that the involvement of *A. pullulans* with any adverse human health condition is extremely rare, more rare in fact, than reports associated with Baker's yeast. Therefore, they attest that the products of *A. pullulans* could be considered generally recognized as safe [Expert Opinion of Ahearn and Ajello, 2001 Appendix 1].

D. Acute Toxicity in Mice and Rats

The Hayashibara Company, Ltd. commissioned three acute studies in rodents to test the toxicity of Pullulan, the production strain of *A. pullulans*, or its lysate (see below). The studies indicate that the product, the organism, or its lysate were not toxigenic or pathogenic to rats or mice, even when administered in doses up to 20g/kg of body weight.

The School of Medicine of Juntendo University conducted two studies in mice in 1974. Only a study summary certificate is available for each study. The number of mice per treatment is not known. One study examined the acute response of mice to a commercial sample of Pullulan and the other study evaluated their response to the production strain of *Pullularia pullulans* (now *Aureobasidium pullulans*). The Pullulan was suspended in olive oil and the organism in water. No deaths were recorded for either study. The LD₅₀ for the organism was determined to be >24.134g/kg body weight, and the LD₅₀ for the product was >14.280g/kg body weight. The investigators indicated that these concentrations were likely the maximum that could be administered, because of the thickness of the preparation [Juntendo University Reports, 1974 Vol 2 Tabs 18 and 19].

A third acute study was performed to note the effects of *A. pullulans* lysate in rats [Mitsubishi Chemical Safety Institute Ltd., 1996 Vol 3

Section IV Safety

Tab 27]. The study was certified as being performed using FDA Guidelines (1982) and US FDA GLP Standards for Nonclinical Laboratory Studies (21 CFR part 58, 1987). *Aureobasidium pullulans* lysate (lot number 960408) was administered orally to ten Sprague-Dawley rats (five weeks old) five per sex at doses of 10 and 20g/kg body weight each. As a positive control *Saccharomyces cerevisiae* lysate (lot number 960408) was also administered to 10 other rats (5 per sex) at a dose of 20g/kg. This treatment served as the positive control. Five additional rats per sex comprised the negative control group, and this group received sterile phosphate-buffered saline. Both the test substance and the positive control were administered at a purity of 66.7% in PBS solution. The Sponsor verified the lysate before administration. Male rats ranged in weight from 135-156g, while females were 116-131g. Five of the 20 females rats weighed less than 120g at treatment, which was the only deviation from the protocol. This was thought to have been caused by the required fasting from the evening before treatment. Since the animals appeared healthy and the difference was only a few grams, they were used for the study. Each animal was identified by body tattoo and cage labels.

Doses of *A. pullulans* lysate and controls amounting to 10 and 20g/kg body weight were divided in two equal aliquots and administered by gastric tube as two separate oral doses 4 hours apart. Food was withheld for about two hours after dosing. Individual animal weights were measured immediately before treatment. The dose volume was 20ml/kg for both the first and second administration.

Animals were observed for mortality and signs of toxicity at approximately 0.5, 1, 3 and 4 hours after the first and second administration. Thereafter, clinical observations were made twice a day for 13 days after administration, except for weekends where only one observation was made each day. On the day of necropsy (14 days after administration) the rats were observed once before anesthetization and sacrificed by exsanguination. Body weights were determined before administration and once a week thereafter. At the

Section IV Safety

conclusion of the study the rats were examined for mortality, body weight, gross clinical signs, gross toxicity of internal organs, and if abnormalities of organs were observed, histopathology would be performed.

No deaths occurred during the study. Body weights increased in a normal fashion and were the same as the negative control. No abnormalities were observed in clinical signs throughout the study. No abnormalities were found in any organs of the control or treated rats. Since gross examination revealed no apparent abnormalities, histopathology of the organs was not performed.

It was concluded that 20g/kg body weight of *Aureobasidium pullulans* lysate administered in two acute oral doses was not lethal or toxic to SD rats, and the LD₅₀ was > 20g/kg body weight [Mitsubishi Chemical Safety Institute Ltd., 1996 Vol 3 Tab 27].

E. Long-term Feeding Study in Rats

Kimoto, *et al.*, published results of a feeding study designed to assess the potential effects of long-term consumption of Pullulan by Sprague-Dawley rats [Kimoto, *et al.*, 1997 Vol 3 Tab 22]. The study was originally conducted by the Department of Public Hygiene, School of Medicine, Juntendo University [Kotani, *et al.*, 1976 Vol 3 Tab 23]. The Pullulan used was taken from a commercial production lot.

One hundred twenty (120) four-week old SD-JCL rats were divided into 4 groups of 30, 15 of each sex. The rats were randomly assigned to one of three treatment groups or a control group. Test groups were administered Pullulan in the diet at levels of 1, 5 and 10% for a period of 62 weeks. Control animals received a standard laboratory diet.

The protocol was designed to include general observations of animal health and activity on a daily basis, and weight determinations on a

Section IV Safety

weekly basis. At the conclusion of the study the rats were anesthetized and blood was collected directly from the heart. Blood samples were analyzed for red and white cell concentrations, differential counts, hemoglobin, and hematocrit. Additionally, the investigators measured concentrations of serum transaminases (AST, ALT), alkaline phosphatase, cholinesterase, the albumin to globulin ratio, total cholesterol, serum protein, and blood sugar. Urine was collected and assayed for protein, sugar, ketones, pH, and occult blood. Animals were exsanguinated and major organs were observed for pathological changes by macroscopic and histologic methods.

The feeding study was originally intended to be for 24 months. However, the study was terminated at 62 weeks due to poor survival resulting from intercurrent pneumonia in all groups, including the control. Examination of all rats that died during the study showed no noteworthy changes other than pneumonia, which accounted for most of the deaths in the colony. The investigators stated that pulmonary abscesses and pneumonia are conditions, which are commonly encountered in long-term studies with mice and rats [Kotani, *et al.*, 1976 Vol 3 Tab 23]. Other investigators who have utilized long-term rat studies to assess the safety of new food ingredients have published reports corroborating respiratory ailments as one of the common findings in older colonies (Woodard, *et al.*, 1973 *Toxicology and Applied Pharmacology* 24, 30-36). In this study all surviving animals were necropsied, and thorough gross post-mortem examinations were conducted after 62 weeks of treatment [Kotani, *et al.*, 1976 Vol 3 Tab 23].

Pullulan did not adversely effect food consumption or food efficiency (See Table 13) [Kimoto, *et al.*, 1997 Vol 3 Tab 22]. The body weight of male rats in every group increased rapidly until the 10th week. This was followed by a gradual increase in weight to about 600 grams at the 40th week, after which weights remained stable. Mean weight gains of the rats fed diets containing 1 and 10% Pullulan were reported to have been somewhat slower than those of the control

Section IV Safety

group; however, these differences were not statistically significant. At the termination of the study, the mean weight of the animals in the 1 and 10% group were significantly less ($P < 0.05$) than the control group. The mean weight of the 5% group was not significantly different. The female rats grew rapidly from the 2nd to the 10th week. Gradual growth was noted until the female rats reached a weight of approximately 350 grams, and no substantial weight gain was noted after the 40th week. The mean body weight gains of all treatment groups were comparable to the controls. None of the mean weights of the treatment groups were significantly different than the control. Therefore there appeared to be no consistent or dose-associated effect of Pullulan consumption on weight gain or absolute weight after 62 weeks [Kotani, *et al.*, 1976 Vol 3 Tab 23 and Kimoto, *et al.*, 1997 Vol 3 Tab 22].

No significant or consistent differences in daily feed intake per animal or per kg body weight were noted between the treatment groups and the control group. No increase in daily intake was measured during the rapid growth period of the 2nd to the 10th week. Therefore, the dietary intake per kg body weight decreased in all groups during this time, and then remained stable throughout the study [Kotani, *et al.*, 1976 Vol 3 Tab 23 and Kimoto, *et al.*, 1997 Vol 3 Tab 22].

Section IV Safety

Table 13
Pullulan Intake

| Dose Level (%in diet) | No. of Rats | | Actual Intake (mg/kg body weight/day) | |
|--------------------------|-------------|----|--|------|
| | ♂ | ♀ | ♂ | ♀ |
| 0 | 15 | 15 | 0 | 0 |
| 1 | 15 | 15 | 480 | 520 |
| 5 | 15 | 15 | 2320 | 2630 |
| 10 | 15 | 15 | 4450 | 5080 |

There were significant differences in hematology and clinical chemistry values of treated rats when compared to the control group; however, these differences were not consistent in relation to Pullulan dose or gender. For the male groups, the mean RBC concentration of the 1 and 10% groups were significantly ($P < 0.01$, 0.05 , respectively) greater than control. The lymphocyte counts of the 1 and 5% groups were less than control ($P < 0.01$, 0.05 , respectively). The 1% treatment group had a significant increase in band neutrophils ($P < 0.01$), while the 5% group had a greater percentage of segmented neutrophils ($P < 0.05$). The 5% treatment group also had a lower ($P < 0.05$) ALT concentration than the control group. The only significant difference between the female groups was that the 5% treatment group had a significantly greater ($P < 0.05$) total cholesterol concentration than the control group. There was no indication of a Pullulan treatment related effect in the animals administered Pullulan at up to 10% of their diet for 62 weeks.

All urine samples were negative for sugar, occult blood or ketones. pH and protein content ranged from 6-8 and + to +++, respectively, without any apparent differences in pattern between the treatment and the control groups.

Section IV Safety

Organ weights were statistically compared on an absolute and per body weight basis. Tables 14 and 15 provide a statistically relevant comparison between the groups for male and female animals. Male rats fed Pullulan at all concentrations showed a decrease in mean liver and right kidney weights when compared to the control group. Other statistically significant differences were also observed (Table 14). However, when organ weights per body weight were compared, none of the differences were significant. The pattern of significant differences of organ weights was not the same in female rats as that observed in males (Table 15). Statistically significant differences ($P < 0.05$) in mean organ weights were calculated between the control and the 10% treatment group for heart, liver, spleen and cecum ($P < 0.01$). However, as with the male groups, when calculated on an organ weight per body weight basis no significant differences were noted. According to several authors, cecal enlargement is a common physiologic response to poorly absorbed sugars and carbohydrates, and is considered an adaptive rather than a pathologic change [Kimoto, *et al.*, 1997 Vol 3 Tab 22; Oku *et al.*, 1979 Vol 3 Tab 33].

Taken together these data suggests that none of the differences were associated with treatment with Pullulan. Additionally, the authors noted that no histologic changes were observed that would indicate that the differences in mean organ weights resulted from a pathologic condition.

Section IV Safety

Table 14
Organ Weights and Organ/Body Weight Ratios in Male Rats Fed for 62 Wks .
(SD) (g)

| | % Pullulan in the diet | | | |
|------------------------------|------------------------|--------|--------|--------|
| | 0 | 1 | 5 | 10 |
| Brain | 1.80 | 2.05 | 2.03 | 1.98 |
| (SD) | (0.26) | (0.12) | (0.11) | (0.10) |
| Brain/bw x 100 | 0.27 | 0.34 | 0.32 | 0.33 |
| Heart | 1.69 | 1.56 | 1.73 | 1.52 |
| (SD) | (0.10) | (0.19) | (0.31) | (0.13) |
| Heart/bw x 100 | 0.26 | 0.26 | 0.27 | 0.25 |
| Liver | 20.8 | 18.0 | 18.44 | 17.6** |
| (SD) | (1.2) | (3.2) | (1.9) | (1.0) |
| Liver/bw x 100 | 3.14 | 3.03 | 2.88 | 2.90 |
| Left lung | 1.13 | 1.12 | 1.00 | 1.12 |
| (SD) | (0.20) | (0.46) | (0.22) | (0.55) |
| Left lung/bw x 100 | 0.17 | 0.19 | 0.16 | 0.18 |
| Right lung | 2.05 | 2.27 | 2.10 | 2.19 |
| (SD) | (0.26) | (0.51) | (0.65) | (0.88) |
| Right lung/bw x 100 | 0.31 | 0.38 | 0.33 | 0.36 |
| Spleen | 1.03 | 0.85 | 1.34 | 0.95 |
| (SD) | (0.22) | (0.16) | (0.79) | (0.18) |
| Spleen/bw x 100 | 0.16 | 0.14 | 0.21 | 0.16 |
| Stomach | 2.51 | 2.29 | 2.37 | 2.24 |
| (SD) | (0.26) | (0.23) | (0.23) | (0.18) |
| Stomach/bw x 100 | 0.38 | 0.38 | 0.37 | 0.37 |
| Testes | 3.53 | 3.59 | 2.92 | 3.52 |
| (SD) | (0.08) | (0.22) | (1.05) | (0.36) |
| Testes/bw x 100 | 0.53 | 0.60 | 0.46 | 0.58 |
| Left kidney | 2.22 | 1.87 | 2.00 | 1.83 |
| (SD) | (0.29) | (0.29) | (0.21) | (0.29) |
| Left kidney/bw x 100 | 0.34 | 0.31 | 0.31 | 0.30 |
| Right kidney | 2.25 | 1.90 | 1.97 | 1.80** |
| (SD) | (0.27) | (0.28) | (0.16) | (0.22) |
| Right kidney/bw x 100 | 0.34 | 0.32 | 0.31 | 0.30 |
| Adrenals | 0.06 | 0.06 | 0.07 | 0.05 |
| (SD) | (0.01) | (0.01) | (0.01) | (0.01) |
| Adrenals/bw x 100 | 0.01 | 0.01 | 0.01 | 0.01 |
| Submandibular gland | 0.81 | 0.77 | 0.74 | 0.64** |
| (SD) | (0.09) | (0.10) | (0.09) | (0.04) |
| Submandibular gland/bw x 100 | 0.12 | 0.13 | 0.12 | 0.11 |

bw = body weight

*P < 0.05; **P < 0.01; Student's t-test.

Section IV Safety

Table 15
Organ Weights and Organ/Body Weight Ratios in Female Rats Fed Pullulan for 62 Wks.
(SD) (g)

| | % Pullulan in the diet | | | |
|------------------------------|------------------------|--------|--------|--------|
| | 0 | 1 | 5 | 10 |
| Brain | 1.75 | 1.65* | 1.54* | 1.62 |
| (SD) | (0.13) | (0.04) | (0.13) | (0.13) |
| Brain/bw x 100 | 0.48 | 0.40 | 0.47 | 0.39 |
| Heart | 1.03 | 1.15* | 1.06 | 1.20* |
| (SD) | (0.11) | (0.13) | (0.12) | (0.14) |
| Heart/bw x 100 | 0.28 | 0.28 | 0.32 | 0.29 |
| Liver | 11.9 | 13.2 | 10.7 | 14.3* |
| (SD) | (2.2) | (3.1) | (1.7) | (2.2) |
| Liver/bw x 100 | 3.25 | 3.22 | 3.23 | 3.43 |
| Left lung | 0.70 | 0.80 | 0.74 | 0.72 |
| (SD) | (0.18) | (0.12) | (0.19) | (0.08) |
| Left lung/bw x 100 | 0.19 | 0.20 | 0.23 | 0.17 |
| Right lung | 1.43 | 1.56 | 1.42 | 1.47 |
| (SD) | (0.50) | (0.41) | (0.37) | (0.12) |
| Right lung/bw x 100 | 0.39 | 0.38 | 0.43 | 0.35 |
| Spleen | 0.55 | 0.61 | 0.53 | 0.75* |
| (SD) | (0.10) | (0.16) | (0.19) | (0.14) |
| Spleen/bw x 100 | 0.15 | 0.15 | 0.16 | 0.18 |
| Stomach | 1.78 | 1.59 | 1.70 | 1.91 |
| (SD) | (0.20) | (0.54) | (0.31) | (0.24) |
| Stomach/bw x 100 | 0.49 | 0.39 | 0.51 | 0.46 |
| Caecum | 1.41 | 1.50 | 1.38 | 2.05** |
| (SD) | (0.27) | (0.36) | (0.30) | (0.45) |
| Caecum/bw x 100 | 0.39 | 0.37 | 0.42 | 0.50 |
| Left kidney | 1.06 | 1.29 | 1.13 | 1.27 |
| (SD) | (0.32) | (0.29) | (0.17) | (0.13) |
| Left kidney/bw x 100 | 0.29 | 0.31 | 0.34 | 0.30 |
| Right kidney | 1.13 | 1.28 | 1.07 | 1.24 |
| (SD) | (0.13) | (0.29) | (0.17) | (0.15) |
| Right kidney/bw x 100 | 0.31 | 0.31 | 0.32 | 0.30 |
| Adrenals | 0.07 | 0.08 | 0.08 | 0.11 |
| (SD) | (0.01) | (0.01) | (0.02) | (0.05) |
| Adrenals/bw x 100 | 0.02 | 0.02 | 0.02 | 0.03 |
| Uterus | 0.85 | 0.98 | 0.86 | 0.93 |
| (SD) | (0.18) | (0.37) | (0.20) | (0.28) |
| Uterus/bw x 100 | 0.23 | 0.24 | 0.26 | 0.22 |
| Ovary | 0.10 | 0.10 | 0.10 | 0.11 |
| (SD) | (0.03) | (0.02) | (0.01) | (0.01) |
| Ovary/bw x 100 | 0.03 | 0.03 | 0.03 | 0.03 |
| Submandibular gland | 0.52 | 0.56 | 0.54 | 0.78 |
| (SD) | (0.08) | (0.05) | (0.07) | (0.44) |
| Submandibular gland/bw x 100 | 0.14 | 0.14 | 0.16 | 0.18 |

bw = body weight;
*P < 0.05; **P < 0.01; Student's t-test.

Section IV Safety

All surviving animals were necropsied and organs were histologically examined. From these examinations it was obvious that an infectious process was occurring throughout the cohort, including control groups. Table 16 provides information on the number and specific types of conditions observed at the termination of the study.

Table 16
Gross Examination and Histopathological Finds Relating to
Pneumonia In Rats at the Termination of the Study

| Surviving Rats (62 wks.) As Numbered in the Original Study | Histopathological Evaluation (from Tables XI and XVIII of the Original Report) |
|--|--|
| 10% Males (5 of 7 surviving) | |
| 1 | Pneumonia (both); Pleural Adhesion (left) |
| 2 | Bronchitis |
| 3 | |
| 4 | |
| 5 | Cecum-sized pulmonary abscess (right) |
| 6 | Pneumonia (right) |
| 7 | Pulmonary abscess (right) |
| 5% Males (3 of 5 surviving) | |
| Note: 4 Reported in Table 8 of the Original Report | |
| 8 | Bronchitis |
| 9 | Grave Pulmonary abscess (right) |
| 10 | |
| 11 | |
| 12 | Pulmonary abscess (right) |
| Males 1% (7 of 9 surviving) | |
| 13 | Pneumonia (left) |
| 14 | Bronchitis |
| 15 | |
| 16 | Pulmonary abscess (right) |
| 17 | Pneumonia (right) |
| 18 | |
| 19 | Pneumonia (right); Pleural Adhesion |
| 20 | Mild Pneumonia |
| 21 | Mild Pneumonia (right) |
| Males Controls (3 of 7 surviving) | |
| 22 | Pulmonary abscess; Pleural Adhesion (both) |
| 23 | |
| 24 | Bronchitis |
| 25 | |
| 26 | Pulmonary abscess (right); Pleural Adhesion (left) |
| 27 | |
| 28 | |

Section IV Safety

(Table 16 Continued)

| | |
|--|---|
| Females 10% (2 of 6 surviving) | |
| 29 | |
| 30 | Bronchitis |
| 31 | Mild pulmonary abscess (both) |
| 32 | |
| 33 | |
| 34 | |
| Females 5% (5 of 10 surviving) | |
| 35 | |
| 36 | Bronchitis |
| 37 | |
| 38 | Pneumonia (left) |
| 39 | Pneumonia (left); Pleural adhesion due to pulmonary abscess (right) |
| 40 | Pulmonary abscess (left); Pneumonia (right) |
| 41 | |
| 42 | |
| 43 | |
| 44 | Mild pneumonia (left) |
| Females 1% (5 of 10 surviving) | |
| 45 | Pleural adhesion due to pulmonary abscess (right) |
| 46 | Bronchitis |
| 47 | |
| 48 | |
| 49 | |
| 50 | |
| 51 | |
| 52 | Pneumonia (right) |
| 53 | Pleural adhesion (left) |
| 54 | Pneumonia (left) |
| Females Control (6 of 13 surviving) | |
| 55 | Bronchitis |
| 56 | |
| 57 | |
| 58 | |
| 59 | |
| 60 | Pleural adhesion (left) |
| 61 | |
| 62 | Pneumonia (right) |
| 63 | |
| 64 | Pulmonary Abscess (left); Pleural adhesion (right) |
| 65 | Pneumonia (left) |
| 66 | |
| 67 | Pneumonia (left) |

Section IV Safety

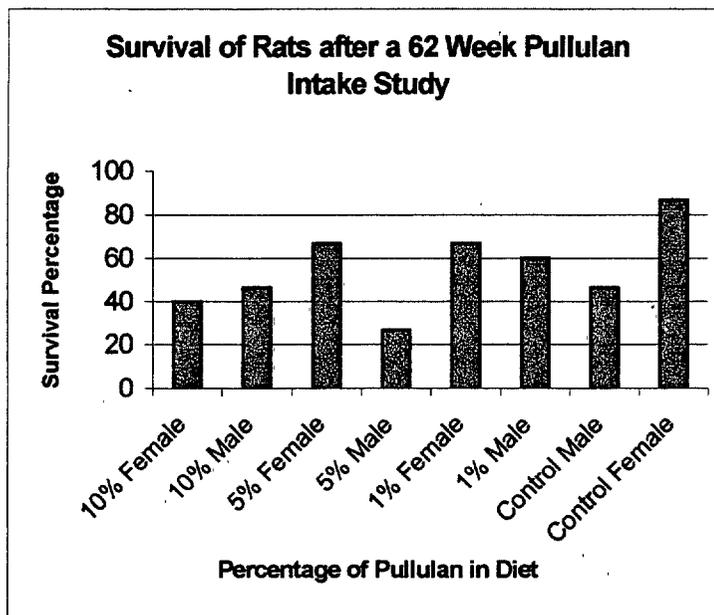
Other than the respiratory related findings (bronchitis, pneumonia, abscesses and adhesions) the following is a list of other conditions found in the various treatment groups. Unless otherwise stated (number of rats), each group represents one animal. Males: Heart: localized myocarditis, 1%, 5% and control: Liver: localized cell infiltration, 10% (3): Spleen: congestion, 1%: Kidney: localized interstitial nephritis, 1% and 5%: Trachea: calcification, 10%: Testicles: hypoplasia of spermatogenesis, 5%. Females: Liver: localized cell infiltration, 1% and 5%; abscesses, control: Spleen: hemosiderosis, 5% and 10%; abscess, control: Adrenals: congestion, 1%, 5%, and control: Uterus: squamous cell metaplasia, 10%; hematoma, 1% and 5%; cyst, control: Abdomen: subcutaneous abscess, 1% (2), 10% and control; myoma, control: Brain: cerebral hematoma, 5%: Inguinal area: myoma, 1%: Neck: myoma, control.

The authors concluded from macroscopic and microscopic examination of selected organs that there was no indication of Pullulan-related toxicity in the organs [Kimoto, *et al.*, 1997 Vol 3 Tab 22].

The study was originally planned to last for 24 months, but because of pneumonia-related deaths in all groups the study was terminated at 62 weeks. Deaths of the treated and control male rats tended to occur relatively early in the study. The survival rate of the male control group was less than 50% at the end of the test (62 weeks). Deaths in female rats were less than those of male rats prior to the 35th week, and more females survived to the end of the study. The investigators indicated that this finding was in accordance with other long-term feeding studies conducted at their facility. Although the survival rate of the 10% Pullulan-fed females was significantly ($P < 0.05$) lower than the control group, no dose dependency was noted between Pullulan intake and survival rate. The survival rate for each group of rats by sex is depicted in the figure below.

Section IV Safety

Figure 2



Study Conclusion

While an ongoing intercurrent disease developed during the study, a comparison of treatment to control groups indicated that Pullulan did not cause any additional untoward effects in the animals. Although individual significant differences in survival rates, and body weights were observed, there was not any variable that was consistent with a dose effect or was seen in both males and females. Some absolute organ weights were significantly different than controls, but when compared on a body weight basis there was no significance. Also, no consistent pattern between males and females was seen. On the basis of the study results, it was concluded that Pullulan lacked toxicological activity. The no-observed-adverse-effect was determined as the highest concentration tested, 10% of the diet. This was equal to or greater than 4450mg/kg body weight/day in males and 5080mg/kg body weight/day in females. The authors stated that these results would support use in various foods at a level of at least 45mg/kg body weight/day [Kimoto, *et al.*, 1997 Vol 3 Tab 22].

Section IV Safety

F. Human Consumption Study

Yoneyama *et al.* investigated the effects of the consumption of reagent grade Pullulan (MW 50,000) on 13 healthy human volunteers. The volunteers were given Pullulan (10g/day) at lunch, either in water or in soup, for fourteen consecutive days. No untoward effects were noted. Some subjects noted a feeling of fullness.

Subjects included 13 healthy adult volunteers (24-53 years old, mean 34.5 years). During the experimental period, the volunteers were allowed to carry out their normal daily activities, but were instructed to avoid the excessive use of alcoholic beverages. There were no other dietary restrictions [Yoneyama *et al.*, 1989 Vol 4 Tab 54].

Six of the 13 volunteers had stool weight, stool pH, short-chain fatty acid (SCFA) concentration, water-soluble saccharide concentration, and the profile of the bacterial flora of the stools tested before and after treatment. Stool weights were determined from samples collected over the 48-hour period just before and after the study period. For blood biochemistry, all 13 subjects were used. The items tested included cholesterol components, lipid components, inorganic salt concentration, hepatic function, and blood sugar level. In addition, blood pressure of all the test subjects was taken.

A reduction in fecal pH as the result of Pullulan intake was detected in 5 of 6 test subjects following stool sampling. The average pH values tended to decrease from pH 6.53 before the study to pH 5.97 after intake. This may be physiologically significant, as various health authorities have linked lowering the pH of the colonic environment to a reduction in the instances of certain cancers [Kritchevsky, 1996 Vol 3 Tab 24]. Correspondingly, total SCFA detected in 1g of feces showed an increase as the result of Pullulan consumption in 5 of 6 test subjects. The average values tended to increase from 6.0mg/g before intake to 8.8mg/g after intake. However, these changes were

Section IV Safety

not found to be statistically significant [Yoneyama *et al.*, 1989 Vol 4 Tab 54].

The daily fecal weight increased 33%; however, the difference was not significant. The results of water-soluble saccharides in the feces of the 6 test subjects showed an average decrease from 135mg/100g of feces to 106mg/100g, although, individual results were highly variable. These data are consistent with essentially complete fermentation of Pullulan in the colon [Yoneyama *et al.*, 1989 Vol 4 Tab 54]. Stool frequency did not change after treatment.

The majority of microorganisms in the human intestine are known to be members of the families, *Bacteroidaceae*, *Bifidobacterium*, *Eubacterium*, and *Peptococcaceae* [Salyers, *et al.*, 1985 Vol 3 Tab 38]. Isolates of all four of these families could be detected in 3 subjects. *Eubacterium* and *Peptococcaceae* were not detected in 1 and 2 subjects, respectively. The number of *Bifidobacterium* increased after Pullulan intake in 5 of 6 subjects. In one test subject, the increase in microorganisms due to Pullulan intake was notable, (> 10,000 fold increase). Members of the *Bacteroidaceae* were the most predominant microfloral organisms in all the test subjects. This population increased, decreased, or showed no change in two each of the 6 test subjects, while, the total numbers of fecal organisms on a log scale (10.8 per 1g of feces) showed no change [Yoneyama *et al.*, 1989 Vol 4 Tab 54]. Rather, there was a demographic shift towards *Bifidobacterium*. Fecal populations of *Bifidobacterium* were shown to increase in five of six subjects over the course of the 14-day study. As a result, the ratio of *Bifidobacterium* to total human fecal microflora increased from 11.9% before consumption of Pullulan to 21.9% after intake. Many health practitioners feel that this is clinically significant, because certain health benefits have been associated with an increase in the population of probiotic bacteria, such as *Bifidobacterium*. A recent Scientific Status Summary published by the Institute of Food Technologists lists several potential and established effects of probiotic bacteria [Institute of Food Technologists, 1999 Vol 2 Tab 14].

Section IV Safety

No significant differences were noted in the 14 serum variables examined before and after treatment. Additionally, no changes were observed in subject blood pressure [Yoneyama *et al.*, 1989 Vol 4 Tab 54].

Some of the test subjects mentioned the sensation of abdominal fullness after taking 10g of Pullulan per day, but no other symptoms were noted. The investigators concluded that there were no adverse effects from the consumption of 10g of Pullulan per day, and that orally administered Pullulan functions as a dietary fiber, which might act to improve the human intestinal environment [Yoneyama *et al.*, 1989 Vol 4 Tab 54].

G. History of Safe Use

HBC Pullulan has been in commercial production since 1976 in Japan. To date more than three thousand metric tons have been sold into the food chain. The product was classified as a food ingredient in Japan by the Food Chemical Section, Environmental Health Department, Ministry of Health and Welfare in 1977 [Official Letters to Hayashibara Company, Ltd. 1976 and 1977 Vol 3 Tab 29]. It is also listed in the Standards for Ingredients of Drugs and is widely used as a pharmaceutical additive for bulking and stabilization of tablets in Japan [Hayashibara Certificate, 1988 Vol 2 Tab 12].

To Hayashibara Company, Ltd's knowledge, no complaints have been made to either the company or the various government entities that would handle such matters. There are at least five agencies in Japan to which consumer complaints concerning food products can be reported. It is likely that any consumer inquiry to a central government agency would be referred to the Japanese Consumer Information Center (JCIC). The JCIC is a nonprofit organization, which was established by the Japanese Government in 1970 to provide consumer education, training programs and publications, test

Section IV Safety

products, alert consumers to potential problems with products, and handle consumer complaints.

The JCIC collects the appropriate information from the consumer, contacts the company, and reviews the available information. A response is formulated, which includes corrective measures. If companies are not responsive to the concerns of JCIC, the case is turned over to appropriate agencies for legal action.

In addition to government-associated agencies in Japan, manufacturers encourage consumer comments directly to them and usually provide a telephone contact number on their product. Since Pullulan is usually used as an ingredient in a food product, it is more likely that any complaints would first be made to the final product manufacturer or to the JCIC. Subsequent to a complaint, Hayashibara would be contacted if there was a question of whether Pullulan was involved in a particular issue.

Although Pullulan has been sold into the Japanese market for more than two decades, no consumer complaints have been reported to any Hayashibara associated company. This provides strong evidence that consumption of Pullulan is safe and has not produced untoward effects based on the concentrations being used in the Japanese food industry.

H. Fate of Pullulan in the Digestive Tract

The determination that Hayashibara Pullulan is safe for human consumption is also based on its digestive pattern. Studies conducted on the fate of Pullulan in the digestive tract have demonstrated that it is hydrolyzed to a very limited extent by the salivary and pancreatic amylases of the upper GI tract, and that essentially no glucose is released during hydrolysis [Okada, *et al.*, 1990 Vol 3 Tab 32]. However, as with many other commonly consumed oligosaccharides, the majority of the ingested Pullulan is

Section IV Safety

fermented by resident bacteria in the large intestine forming short-chain fatty acids [Nakamura, 1984 Vol 3 Tab 28].

Glucose polymers may differ in the site(s) of digestion based on their structure and molecular weight (Murray, *et al.*, *Journal of Nutrition* 128 (11):2032-5, 1998 and (Flickinger EA, *et al.* *Journal of Nutrition* 130:1267-73, 2000). Nonetheless, there is no indication that any glucooligosaccharides are in and of themselves toxic to humans. Hayashibara International Inc. sought an Expert Opinion from Dr. George Fahey, of the Department of Animal Sciences, University of Illinois, regarding the relationship of structure to the digestibility and safety of glucose polymers.

Dr. George Fahey stated that, "glucose-based oligosaccharides are common in the human diet, and exhibit a wide variety of structural variations. While these structural variations may affect the site (small intestine vs. colon) and extent of digestion, none are known to be harmful to humans, and many glucose polymers have been tested in a clinical setting. Using starch as an example, it is well known that rate of digestion is effected by the relative amounts of amylose and amylopectin in the starch fraction. Goddard *et al.*, (1984) explained that the rate of amylose digestion is slower than that of amylopectin, because it is a linear molecule, whose glucose units participate more readily in hydrogen bonding than do those of the more highly branched amylopectin. This tends to make them less accessible to enzymatic digestion, according to Thorne, *et al.*, (1983). Further, amylopectin is a larger molecule, with more surface area for enzymatic attack.

The structure of starch, and therefore its digestibility may be modified purposely or inadvertently through food preparation. For instance, Annison and Topping (1994) reported that it was possible to physically modify the structure of starch by retrogradation, so that it became partially inaccessible to enzymatic attack. This type of starch is now known as resistant starch.

Section IV Safety

Vonk, *et al.* (2000), found that highly digestible (80%) cornstarch contained 26% amylose and 74% amylopectin, whereas resistant cornstarch contained 62% amylose and 38% amylopectin. This and other studies have shown that the digestibility of resistant starch drops to about 50%. Likewise, maltodextrins are generally considered to be highly digestible sources of carbohydrate energy, however, the addition of heat and enzymatic hydrolysis during preparation of maltodextrins can create a greater variety of bond formations, including β -1-3, β -1-4 and β -1-6 linkages. The glucose digestibility of these modified maltodextrins has been shown to be lower than unmodified maltodextrin.

Clinical studies both in our laboratories and elsewhere have shown that a range of glucose polymer structures have been well tolerated by animal or human subjects, and no effects adverse to health have been noted. Pullulan is also a glucose polymer and its linear structure with limited branching appears to reduce its digestibility. However, there is no indication that this particular structure would be less well tolerated than any of those already discussed."

As noted by Dr. Fahey, Pullulan is structurally similar to amylose and therefore more resistant to digestion in the upper gastrointestinal tract than typical cornstarch derived maltodextrins or native food grade starches. While Okada, *et al.*, found that enzymatic hydrolysis of Pullulan stops at the α -1,6-linked bonds, the normal human diet contains tens, if not hundreds of grams per day of α -1,6-linked glucose units in common foods [Okada, *et al.*, 1990 Vol 3 Tab 32 and Southgate, 1998 Vol 4 Tab 40]. Consumption of Pullulan should not substantially alter current consumption patterns because, in large part, Pullulan will displace other carbohydrates in the diet, which also contain glucose linked by α -1,6 or α -1,4-glycosidic units [Enns, *et al.*, 1997 Vol 2 Tab 8 and Southgate, 1998 Vol 4 Tab 40].

Okada *et al.* designed an *in vitro* study to simulate the digestion of Pullulan. Two Pullulan products were tested including a commercially available Pullulan. Pullulan (PI-20) had a mean

Section IV Safety

molecular weight of 200,000 and contained about 8% low molecular weight (<10,000) sugars lacking the structure of Pullulan. The second sample was a reagent grade Pullulan (PR-5), with a mean molecular weight of 50,000, but containing no low molecular weight sugars. The PI-20 product was not degraded by gastric fluid, but was partially converted to reducing sugars by salivary and pancreatic enzymes (an increase of 0.6% and 0.7%, respectively). Subsequently, a rat-derived small intestine enzyme system partially hydrolyzed Pullulan PI-20, liberating 9.7% reducing sugar. Conversely, gastric acid, salivary or pancreatic enzymes did not hydrolyze the PR-5 sample; however, treatment with rat small intestine enzymes did result in an increase of 3.6% of reducing sugars, suggesting that most of the increase in reducing sugar came from the hydrolysis of the low molecular weight saccharides in the PI-20, and not from breakdown of the Pullulan itself.

Further examination of Pullulan digestion demonstrated that for samples with a mean molecular weight greater than approximately 65,000, the amount of glucose formed is constant at about 1.5%. For preparations with mean molecular weights less than this, the amount of glucose formed increases in a constant manner with decreased molecular weight. For samples with molecular weights of 48,000, 5,800 and 990 the amount of glucose formed from enzymatic digestion was 1.9, 8.6 and 36.3%, respectively [Okada, *et al.*, 1990 Vol 3 Tab 32].

Microbial fermentation of Pullulan was examined using an *in vitro* fecal culture system that showed that Pullulan was fermented to short-chain fatty acids in a manner typical of dietary carbohydrates that are not digested by hydrolytic enzymes. About 50% of the administered Pullulan was converted to short chain fatty acids (SCFA), mainly acetic, propionic and n-butyric acids. This, again is consistent with the other common dietary fermentable sugars and polysaccharides [Okada, *et al.*, 1990 Vol 3 Tab 32].

Section IV Safety

The next step in the multi-part study of Okada, *et al.*, was to determine the energy contribution of Pullulan. This study used a Pullulan with a mean molecular weight of 50,000. In the study, the assumption was made that all glucose produced in the small intestine was absorbed so that there was an energy transfer of 100%. Additionally, it was assumed that all the SCFA were absorbed in the colon. The amount of glucose absorbed from 1g of Pullulan would be about $0.027\text{g} \times 3.74\text{kcal/g} = 0.10\text{kcal}$. In order to determine the amount of energy produced by the SCFAs, the amount of SCFAs that were present after all the Pullulan had disappeared (after 8 hours of *in vitro* fermentation) was determined. The energy derived from the SCFAs was calculated from an equation developed by the authors showing that 1g Pullulan resulted in 2.05kcal [Okada, *et al.*, 1990 Vol 3 Tab 32].

In this study the amount of Pullulan reaching the colon was 0.976g. Therefore, the energy derived from the large intestine was $2.05\text{kcal/g} \times 0.976\text{g} = 2.00\text{kcal}$. The final calculation showed that the energy produced per gram of ingested Pullulan was: 0.10kcal (from glucose) + 2.00kcal (from SCFA) = 2.10kcal .

The authors concluded that Pullulan products are dietary fibers that are not susceptible to the normal enzymes of the gastrointestinal tract.

I. Systemic Exposure

No data are available to suggest that Pullulan is or is not assimilated into the body after ingestion. Because of the size of the Pullulan molecule and the minimal enzymatic digestion that occurs prior to the large intestine, the systemic uptake of Pullulan is highly unlikely [Weiner, 1988 Vol 4 Tab 52].

The pharmacokinetics of Pullulan in the body was examined by intravenous injection of male Wistar rats with fluorescein-labeled Pullulan (MW 58,200) [Kaneo, *et al.*, 2001 Vol 3 Tab 20]. Animals were fasted for 16 hours, anesthetized, and injected with a single

Section IV Safety

0.2ml dose of labeled Pullulan through the jugular vein. Samples of blood (0.3ml) were obtained periodically to track the clearance of Pullulan. Results showed that intravenously administered Pullulan was rapidly eliminated from the blood circulation in a dose dependent manner, followed by an appreciable distribution to the liver.

The data suggested that even if Pullulan entered the circulatory system from the gut, it would clear the blood stream in a relatively short period of time. The authors concluded from this and other studies that Pullulan is not toxic and lacks immunogenicity when administered intravenously [Kaneo, *et al.*, 2001 Vol 3 Tab 20].

J. Mutagenicity of Pullulan

The mutagenicity of pullulan was assessed with and without metabolic activation in *Salmonella typhimurium* strains (TA100, TA1535, TA98 and TA1537). Pullulan did not increase the number of revertants per plate in any strain at any dose, including 10,000 µg/plate with or without metabolic activation, from microsomal enzymes from Aroclor-induced rat liver. These data suggest that Pullulan lacks mutagenic/carcinogenic potential. Further, Pullulan was not toxic to rats when given at levels of up to 10% of the diet [Kimoto, *et al.*, 1997 Vol 3 Tab 22].

K. Conclusions on Safety

Hayashibara has provided information on the chemical composition of Pullulan, its elaboration by a non-pathogenic, non-toxigenic organism, *Aureobasidium pullulans*, the manufacturing and purification process for HBC Pullulan, and a set of food grade specifications. Results of both animal toxicology studies, human feeding studies, and classic mutagenicity studies have shown that even when HBC Pullulan is fed at levels up to 10% of the diet, no adverse effects, and no deaths attributable to treatment were observed [Kimoto, *et al.*, 1997 Vol 3 Tab 22]. Further, systemic exposure of Pullulan to rats resulted in no adverse effects. Humans

Section IV Safety

consuming HBC Pullulan in an amount of 10 grams per day at a single eating occasion for 14 consecutive days reported sensations of fullness, but no adverse observations were recorded, and there was no change in blood biochemistry over the course of the study [Yoneyama, *et al.*, 1989 Vol 4 Tab 54]. Finally, in 20 years of consumption by humans in Japan, where HBC Pullulan has been eaten in a number of food products has not produced a known complaint related to safety.

The amount in the human study (10g/day) is nearly equal to the very conservative estimated mean intake reported in Section III Table 12 (9.41g/d/person). Dr. George C. Fahey, Jr. of the University of Illinois provided an Expert Opinion on the safety of consuming HBC Pullulan at levels equivalent to those recommended for soluble fiber intake (25g/1000 kcal) for persons with diabetes [Vinik, *et al.*, 1987 Vol 4 Tab 50; and Fahey, Expert Opinion, 2001 Appendix 1]. The conclusion of the Expert Opinion was that HBC Pullulan, when consumed at a concentration of 25g/1000 kcal would have the same physiological effects as any other source of soluble fiber, and therefore should pose no threat to human health.

A second Expert Opinion by Drs. Donald Ahearn and Libero Ajello addressed the issue of safety of products prepared from the source organism, *Aureobasidium pullulans* [Ahearn and Ajello, Expert Opinion, 2001 Attachment A]. The conclusion of the Expert Opinion was that products produced from *Aureobasidium pullulans* could be considered safe.

While the fact that the organism is not pathogenic provides reassurance from a theoretical point of view, as a practical matter, the organism does not come into contact with the final Pullulan product. The company's system of Critical Controls specifies that each lot be tested for any viable *Aureobasidium pullulans*. Written process controls state that any lot containing the source organism be destroyed (See Section II). The sterility of the Pullulan product (See Tables 4 and 5, Section II) indicates that the manufacturing process

Section IV Safety

eliminates *Aureobasidium pullulans*, and other non-pathogenic organisms, providing for a safe product.

Based on the data and information described above, the Expert Opinions commissioned by the company, and more than twenty years of safe use in Japan, Hayashibara has concluded that HBC Pullulan may be considered generally recognized as safe under scientific procedures for its intended use in food, when manufactured and used in accordance with current Good Manufacturing Practices.

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August 20, 2001



MEMORANDUM

TO: Alan B. Richards, Ph.D.
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FROM: Donald G. Ahearn, Ph.D.
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Libero Ajello, Ph.D.
Guest Researcher
National Center for Infectious Diseases
Centers for Disease Control and Prevention
Division of Bacterial and Mycotic Diseases
Mycotic Diseases Branch

SUBJECT: Safety of *Aureobasidium pullulans*

Aureobasidium pullulans (de Bary) Arnaud is a common black saprobic mould with a world-wide distribution in both indoor and outdoor environments. As a member of the phyllosphere community and a common biofilm member on painted surfaces and shower curtains, it is inhaled and ingested with fresh fruits and vegetables on a daily basis. Prior to the mid-1980's, the species was associated occasionally with superficial infections in humans, but many of these reports have been considered questionable (McGinnis, M. 1980, Laboratory handbook of medical mycology. Academic Press Inc., N.Y.). Early clinical studies either failed to establish a pathogenic association or the taxonomic procedures failed to distinguish their isolates from *Exophiala* spp. In the past several decades there have been a few additional reports (e.g. see Salkin et al. 1986. J. Clin. Microbiol. 23:826) on the pathogenicity of *A. pullulans* for seriously immunocompromised patients, a phenomenon that is considered possible for most fungi including the baker's yeast *Saccharomyces cerevisiae*. Indeed there are far more reports associating this beneficial and safe industrial yeast with various disease syndromes than the rare associations indicated for *A. pullulans*.

Host debilitation is by far the primary factor in the opportunistic or adventitious involvement of saprobic fungi with humans. Nevertheless, in our over 30 years of experience with yeasts and moulds in environmental, industrial and clinical settings, the involvement of *A. pullulans* with any adverse human health related problems is extremely rare.

We recommend on the basis of our experiences and our knowledge of the extensive laboratory and industrial applications of *A. pullulans* that the species and its products be recognized under GRAS status.

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September 17, 2001

Ms. Lee B. Dexter
Lee B. Dexter & Associates, Technology Consultants
15704 Webberville Road
Austin, TX 78724

Dear Lee:

Thank you for the opportunity to provide an opinion regarding the Tolerable Upper Intake Level (UL) for pullulan manufactured by Hayashibara Company Ltd., Okayama, Japan. This is a timely issue as the UL for dietary fiber, broadly defined, will be reviewed in the upcoming report that will provide Dietary Reference Intakes (DRIs) for macronutrients. I was a member of the "Panel on the Definition of Dietary Fiber" that wrote the report entitled "Dietary Reference Intakes: Proposed Definition of Dietary Fiber" that will serve as the basis for the recommendation on fiber that will be published in the macronutrient report. That report is scheduled to be published at the end of this year. To this point in time, potential adverse health effects of dietary fiber are not known but, when necessary, each fiber is considered on a case-by-case basis.

From the information that I have reviewed, I conclude that pullulan consumption is acceptable when consumed by humans at the level of 25 grams/1,000 kcal. Indeed, both the American Medical Association and the American Dietetic Association recommend that the total daily intake of dietary fiber should be as high as 35 grams/day. The American Diabetes Association published a paper in 1987 indicating that a 25 gram/1,000 kcal level is desirable for human diabetics.

In my 25 years of professional experience in the area of dietary fiber, the only negative effect of fiber consumption that I have come across is in developing countries where largely unrefined diets are fed and where mineral absorption and retention issues may surface as a result of the poor nutrition experienced by the populations residing there. But in the case of Western man-eating more refined foods, many of them supplemented with minerals, fiber appears to have no untoward effects on mineral absorption and retention.

As regards pullulan itself, it is a water soluble alpha-glucan containing alpha-1,4 and alpha-1,6-linked glucose units with a stair-step structure. Japanese researchers fed rats diets containing as much as 10% by weight of pullulan and found that pullulan lacked toxicological activity as demonstrated by an entire battery of specific tests.

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The issue is occasionally raised as to whether one can consume too much fiber. Organoleptic properties of the fiber itself generally will prevent its over-consumption by humans. By themselves, many are dry, dusty, or gritty, and it is often the challenge of the food preparation specialist to mask their properties when included in complete foods. Thus, maximum levels of incorporation of fibers into many different types of food products, including enteral formulas, have been defined. These levels nearly always are below the 35 grams/day quantity recommended by the various medical agencies.

As to whether there are adverse events that might occur if humans consumed a high quantity of pullulan, gas production during the early stages of consumption might occur. But colonic microbes usually will adapt to high level fiber feeding after 4 to 7 days, after which gas production will be reduced to normal levels. In addition, humans may adapt to high level fiber feeding by gradually increasing their fiber consumption over a 7 day period. This generally will circumvent the problem outlined above. Pullulan should be no different than any other fiber in this regard.

In summary, I see no problem with pullulan feeding at levels approximating 25 grams/1,000 kcal. Given its chemical structure, its rate and extent of digestion should be relatively high, it should exert no untoward effects on the microbial ecosystem of the large bowel or on the morphology of the organ itself, and no negative systemic effects should be demonstrated as a result of its consumption.

Sincerely,

[Redacted signature box]

George C. Fahcy, Jr.
Professor
Animal Sciences/Nutritional Sciences

GCF/hs

[Redacted stamp]
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Appendix I
Expert Opinions

Hayashibara Pullulan Expert Panel Opinion

Introduction

The undersigned, an independent panel of recognized experts (Expert Panel), qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was convened by Hayashibara International Inc. to determine the Generally Recognized As Safe (GRAS) status of Hayashibara Pullulan for use as a food ingredient. The resumes of each Panelist are on file at the offices of Hayashibara International Inc. in Westminster, Colorado. The qualifications of the Panel satisfy the requirements set forth in the Federal Food, Drug, and Cosmetic Act's definition of generally recognized as safe (GRAS) substances (§ 201(s)) and 21 CFR 170.30(a) "Eligibility for classification as generally recognized as safe (GRAS)".

A comprehensive search of the scientific literature for information on the safety/toxicity of Pullulan and Hayashibara Pullulan through September 2001 was undertaken by Lee Dexter and Associates and was made available to the Expert Panel. The members of the Expert Panel independently evaluated information provided by Hayashibara and other materials deemed appropriate or necessary. Following this independent and critical evaluation, the Expert Panel conferred by telephone and met in Washington, D.C. on July 25, 2001, with representatives of Hayashibara International Inc., who presented additional pertinent safety and functionality information associated with Hayashibara Pullulan. The information critically evaluated by the Panel was compiled into a GRAS Report, which supports the eligibility of Hayashibara Pullulan as a GRAS ingredient in accordance with 21 CFR 170.36. The information provided for the Panel's review was presented in the form stipulated in 21 CFR 170.35. The Panel unanimously agreed to the GRAS status of Hayashibara Pullulan as a food ingredient when produced and used in accordance with current Good Manufacturing Practices and meeting the specifications described herein.

Notice of GRAS exemption claim:

Hayashibara Pullulan is exempt from premarket approval requirements of the Federal Food, Drug and Cosmetic Act because it has been determined to be generally recognized as safe (GRAS) under conditions of intended use by experts qualified by scientific training and experience.

A Summary of the Basis of the GRAS Determination

Chemistry and Manufacturing

- Hayashibara Pullulan is a linear α -D-glucan comprised of regular repeating trisaccharide units [Catley, *et al.*, 1986 Vol 2 Tab 7].
- The molecular weight range for commercial Pullulan may range from 50,000 to 5,000,000 daltons, depending upon the conditions under which the organism is grown [Sugimoto, 1978 Vol 4 Tab 55, Ueda *et al.*, 1963 Vol 4 Tab 61, and Catley, *et al.*, 1986 Vol 2 Tab 7]. Regardless of the molecular weight the structure is identical.
- Structurally, Pullulan is a linear α -D-glucan comprised of regular repeating trisaccharide units. These maltotrioses (in which three glucose units are linked through 1,4-glucosidic bonds) are in turn terminally linked to a series of other maltotrioses through α 1,6-glucosidic bonds creating a long stair-step-type structure [Catley, *et al.*, 1986 Vol 2 Tab 7]. A small percentage of maltotetrose units can also be incorporated into the structure.
- Hayashibara Pullulan is produced under current Good Manufacturing Practices.
- Hayashibara Pullulan is produced using a non-pathogenic and non-toxicogenic strain of *Aureobasidium pullulans*. The organism is cultured and the Pullulan is excreted extracellularly. The culture fluid undergoes several steps to remove impurities and concentrate the Pullulan into the final product.
- Hayashibara Pullulan occurs as a white to slightly yellowish powder, depending on the extent of deionization during processing. Pullulan products designated PI have undergone extensive deionization, and are white in color. Pullulan, which has been purified and designated as food grade (PF) may be white to slightly yellowish.

Final product specifications for Hayashibara Pullulan are shown below:

Final Product Specifications of HBC Pullulan PF-20

| Variable | Specification |
|--------------------------------|--|
| Appearance | White to slightly yellowish powder, tasteless and odorless |
| Pullulan purity (dry basis) | > 90% |
| Loss on drying | < 6.0% |
| Residue on ignition | < 1.5% |
| Viscosity (10 wt%, 30°C) | 100 - 180 mm ² /s |
| Lead | < 0.1ppm |
| Arsenic | < 2 ppm |
| Heavy metals | < 5 ppm |
| pH | 5.0-7.0 |
| Mono, di- and oligosaccharides | < 10% (dry basis) |
| Coliforms | < 10/g maximum |
| Yeast and molds | < 100/g maximum |
| <i>Salmonella sp.</i> | Negative/25 g |
| <i>E. coli</i> | Negative/25 g |
| <i>Staphylococcus aureus</i> | Negative/25 g |

Final Product Specifications of HBC Pullulan PF-10

| Variable | Specification |
|--------------------------------|--|
| Appearance | White to slightly yellowish powder, tasteless and odorless |
| Pullulan purity (dry basis) | > 90% |
| Loss on drying | < 6.0% |
| Residue on ignition | < 5.0% |
| Viscosity (10 wt%, 30°C) | 15-25 mm ² /s |
| Lead | < 0.1ppm |
| Arsenic | < 2 ppm |
| Heavy metals | < 5 ppm |
| pH | 5.0-7.0 |
| Mono, di- and oligosaccharides | < 10% (dry basis) |
| Coliforms | < 10/g maximum |
| Yeast and molds | < 100/g maximum |
| <i>Salmonella sp.</i> | Negative/25g |
| <i>E. coli</i> | Negative/25g |
| <i>Staphylococcus aureus</i> | Negative/25g |

- An analysis of five lots of Hayashibara Pullulan demonstrates that the specifications can be consistently met.
- Chemical and heavy metal analyses of Hayashibara Pullulan products demonstrate Hayashibara Pullulan does not contain toxicants of concern; for example, lead levels are less than 0.1ppm (the LOD).

Use and Functionality

- Pullulan is a glucose polysaccharide, as are other common food grade ingredients such as starch, dextrin and maltodextrin. All four substances consist of glucose units linked through α -1,4-glucosidic and α -1,6-glucosidic bonds.
- Both dextrans and Pullulan are similar to amylopectin molecules that are a main component of food starches.
- Pullulan has been used in Japan for more than 20 years, a history of safe use that supports its safety.
- Pullulan is used as both an indirect food ingredient for coatings on food packaging and as a direct food additive in a variety of applications.
- In 1977, Japanese regulatory authorities approved the use of Pullulan in all food applications. Pullulan was also approved as an auxiliary medical additive and listed in *Non-Official Drugs Specifications* of the Pharmacopoeia of Japan in 1986.
- Pullulan may be used in foods for a variety of Technical Effects, which are codified at 21 CFR §170.3 (o) (1-32):
 - (8) "Emulsifiers and emulsifier salts"
 - (14) "Formulation aides"
 - (16) "Humectants"
 - (20) "Nutrient supplements"
 - (24) "Processing aids"
 - (28) "Stabilizers and thickeners"
 - (29) "Surface-active agents"
 - (31) "Synergists"
 - (32) "Texturizers"
- Dietary fiber content (on an as is basis) of five commercial lots of Hayashibara

Pullulan ranged from 67.5 to 69.5% for PI-20 and from 59.2 to 65.7% for PF-20.

- The estimated daily mean exposure derived from calculations based on USDA's *Continuing Survey of Food Intakes by Individuals* (1996) is 9.41 grams of HBC Pullulan per day. The 90th percentile is then estimated to be 18.82 grams per day.

Safety Studies

Safety studies in animals and a history of safe use in Japan (>20 years) support the safety of Hayashibara Pullulan.

Similarity to Other Foods with a History of Safe Use

- Pullulan is virtually identical in composition to maltodextrin (which has been affirmed GRAS under 21 CFR Part 184), and to the amylopectin component of food grade starches. All three substances consist of glucose units linked through α -1,4- and α -1,6-glucosidic bonds. A comparison of the percentage of α -1,6-glucosidic bonds was presented by Hayashibara International Inc.

**Comparison of the Percent of α 1,6-Glucosidic Bonds
in Common Polyglucoses**

| | |
|--------------|-----|
| Pullulan | 30% |
| Maltodextrin | 20% |
| Cornstarch | 5% |

- Pullulan is similar to other polyglucoses in structure, and the hydrolytic enzymes that degrade it are common to those that hydrolyze other polyglucoses. Therefore, the ultimate degradation product from Pullulan fermentation is glucose.

Physiological Function as a Dietary Fiber

- Hayashibara Pullulan is a dietary fiber, which functions physiologically as an indigestible polysaccharide [Oku, *et al.*, 1979 Vol 3 Tab 43].
- Studies conducted on the fate of Pullulan in the digestive tract have demonstrated that it is only partially hydrolyzed by the salivary and pancreatic amylases of the upper GI tract, and that essentially no glucose is released during hydrolysis by these enzymes.
- The majority of Pullulan is fermented in the large intestine to short chain fatty acids [Nakamura, *et al.*, 1984 Vol 3 Tab 40]. However, very small quantities of glucose may be released from Pullulan by the action of enzymes in the small intestine [Okada, *et al.*, 1990 Vol 3 Tab 42].
- The difference in the digestibility of Pullulan in comparison to other carbohydrates, such as starch, may be attributed to the differing percentages of α -1,6-glucosidic bonds. Since Pullulan contains a higher percentage of α -1,6 bonds, it is more resistant to digestion in the upper gastrointestinal tract [Okada, *et al.*, 1990 Vol 3 Tab 42 and Kimoto, *et al.*, 1997 Vol 2 Tab 22].
- Normal western diets contain tens to hundreds of grams per day of α -1,6-linked glucose units in common foods [Enns, *et al.*, 1997 Vol 2 Tab 11].
- Dr. George C. Fahey, Jr. of the University of Illinois provided an Expert Opinion

on the safety of consuming HBC Pullulan at levels equivalent to those recommended for soluble fiber intake (25g/1000 kcal) for persons with diabetes [Vinik, *et al.*, 1987 Vol 4 Tab 62; and Fahey, Expert Opinion, 2001 Attachment A]. Dr. Fahey stated that, "As to whether there are any adverse events that might occur if humans consumed a high quantity of pullulan, gas production during the early stages of consumption might occur. But colonic microbes usually will adapt to high level fiber feeding after 4 to 7 days, after which gas production will be reduced to normal levels. In addition, humans may adapt to high level fiber feeding by gradually increasing their fiber consumption over a 7-day period. This generally will circumvent the problem outlined above. Pullulan should be no different than any other fiber in this regard." Dr. Fahey concluded that, "Given its chemical structure, its rate and extent of digestion should be relatively high, it should exert no untoward effects on the microbial ecosystem of the large bowel, or on the morphology of the organ itself, and no negative systemic effects should be demonstrated as the result of its consumption."

Safety of the Source Organism

- Drs. Donald Ahearn and Libero Ajello provided an Expert Opinion covering products prepared from the source organism, *Aureobasidium pullulans* [Ahearn and Ajello, Expert Opinion, 2001 Attachment A]. The conclusion of the Expert Opinion was that, "Host debilitation is by far the primary factor in the opportunistic or adventitious involvement of saprobic fungi with humans. Nevertheless, in our over 30 years of experience with yeasts and moulds in environmental, industrial and clinical settings, the involvement of *A. pullulans* with any adverse human health related problems is extremely rare. We recommend on the basis of our experiences and our knowledge of the extensive laboratory and industrial applications of *A. pullulans* that the species and its products be recognized under GRAS status." The Experts also stated that reports linking *Aureobasidium pullulans* to any pathological incident in humans were more rare than those for the common industrial yeast, *Saccharomyces cerevesiae*.

Safety Studies

- Acute studies in mice and rats indicated that neither *Aureobasidium pullulans* nor its lysate were toxigenic at doses up to 20 g/kg body weight.

- The oral LD₅₀ of Pullulan in mice was reported to be >14.280g/kg body weight. The investigators claimed that additional material could not be administered, due to the thickness of the preparation [Juntendo University Reports, 1974 Vol 4 Tabs 80 and 81].
- Kimoto, *et al.*, reported on a study designed to assess the potential long-term toxicity of Pullulan in Sprague-Dawley rats [Kimoto, *et al.*, 1997 Vol 2 Tab 22]. Test groups were administered Pullulan at levels up to 10% of the diet. The study was terminated at 62 weeks due to intercurrent pneumonia in all groups.
 - There were no consistent treatment-related dose-dependent adverse effects reported on any of the parameters evaluated including food consumption, food efficiency, body weight gain, clinical chemistry, hematology, organs weights, gross and microscopic pathology.
 - The no-observed-adverse-effect level (NOAEL) was the highest concentration tested, 10% of the diet, equal to 4450 mg/kg body weight/day in males and 5080 mg/kg body weight/day in females.
- The authors stated that, "A no-observed-adverse-effect level of 10% in the diet, the highest concentration tested, equal to 4450 mg/kg body weight/day, will support an acceptable daily intake of at least 45mg/kg body weight/day as an ingredient in food" [Kimoto, *et al.*, 1997 Vol 2 Tab 22].

Digestion and Fermentation of Pullulan in Humans

- Yoneyama *et al.*, investigated the effects of Hayashibara Pullulan (m.w. 50,000) by thirteen healthy human volunteers who consumed 10 grams of Pullulan/day at lunch, either in water or in soup, for fourteen days.
 - Analysis of stool samples revealed that Pullulan was completely fermentable by human intestinal bacteria, that no residual Pullulan was detected in the feces, that daily stool weight for those subjects increased by 33%, and that the mean fecal pH levels decreased [Yoneyama *et al.*, 1989 Vol 4 Tab 72]. These results support the physiological functionality of Pullulan as a soluble fiber in the human gastrointestinal tract.

- Fecal populations of *Bifidobacterium* were shown to increase in five of the six subjects over the course of the 14-day study. The ratio of *Bifidobacterium* to total human fecal microflora increased from 11.9% before consumption of Pullulan to 21.9% after intake.
 - No significant differences were observed in serum enzymes, electrolytes, neutral and total fats, total LDL, HDL-cholesterol, phospholipids, or beta-lipoprotein [Yoneyama *et al.*, 1989 Vol 4 Tab 72].
 - Abdominal fullness was the only symptom reported by several subjects.
- Studies by Okada, *et al.*, established that the energy contribution of SCFA derived from the fermentation of 1g of Pullulan by human fecal bacteria was 2.00kcal [Okada, *et al.*, 1990 Vol 3 Tab 42].

Conclusion

Based on its independent and collective critical evaluation of the information and data summarized in the GRAS Report, the Panel concluded that Hayashibara Pullulan, meeting the specifications described herein and produced and used in accordance with cGMP, is Generally Recognized As Safe (GRAS) by scientific procedures for use as a food ingredient at the levels cited in the Report in various major food categories.

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Dr. Michael P. Doyle
Professor of Microbiology
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August 20, 2001



MEMORANDUM

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FROM: Donald G. Ahearn, Ph.D.
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Libero Ajello, Ph.D.
Guest Researcher
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SUBJECT: Safety of *Aureobasidium pullulans*

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Host debilitation is by far the primary factor in the opportunistic or adventitious involvement of saprobic fungi with humans. Nevertheless, in our over 30 years of experience with yeasts and moulds in environmental, industrial and clinical settings, the involvement of *A. pullulans* with any adverse human health related problems is extremely rare.

We recommend on the basis of our experiences and our knowledge of the extensive laboratory and industrial applications of *A. pullulans* that the species and its products be recognized under GRAS status.

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September 17, 2001

Ms. Lee B. Dexter
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Dear Lee:

Thank you for the opportunity to provide an opinion regarding the Tolerable Upper Intake Level (UL) for pullulan manufactured by Hayashibara Company Ltd., Okayama, Japan. This is a timely issue as the UL for dietary fiber, broadly defined, will be reviewed in the upcoming report that will provide Dietary Reference Intakes (DRIs) for macronutrients. I was a member of the "Panel on the Definition of Dietary Fiber" that wrote the report entitled "Dietary Reference Intakes: Proposed Definition of Dietary Fiber" that will serve as the basis for the recommendation on fiber that will be published in the macronutrient report. That report is scheduled to be published at the end of this year. To this point in time, potential adverse health effects of dietary fiber are not known but, when necessary, each fiber is considered on a case-by-case basis.

From the information that I have reviewed, I conclude that pullulan consumption is acceptable when consumed by humans at the level of 25 grams/1,000 kcal. Indeed, both the American Medical Association and the American Dietetic Association recommend that the total daily intake of dietary fiber should be as high as 35 grams/day. The American Diabetes Association published a paper in 1987 indicating that a 25 gram/1,000 kcal level is desirable for human diabetics.

In my 25 years of professional experience in the area of dietary fiber, the only negative effect of fiber consumption that I have come across is in developing countries where largely unrefined diets are fed and where mineral absorption and retention issues may surface as a result of the poor nutrition experienced by the populations residing there. But in the case of Western man eating more refined foods, many of them supplemented with minerals, fiber appears to have no untoward effects on mineral absorption and retention.

As regards pullulan itself, it is a water soluble alpha-glucan containing alpha-1,4 and alpha-1,6-linked glucose units with a stair-step structure. Japanese researchers fed rats diets containing as much as 10% by weight of pullulan and found that pullulan lacked toxicological activity as demonstrated by an entire battery of specific tests.

000117

The issue is occasionally raised as to whether one can consume too much fiber. Organoleptic properties of the fiber itself generally will prevent its over-consumption by humans. By themselves, many are dry, dusty, or gritty, and it is often the challenge of the food preparation specialist to mask their properties when included in complete foods. Thus, maximum levels of incorporation of fibers into many different types of food products, including enteral formulas, have been defined. These levels nearly always are below the 35 grams/day quantity recommended by the various medical agencies.

As to whether there are adverse events that might occur if humans consumed a high quantity of pullulan, gas production during the early stages of consumption might occur. But colonic microbes usually will adapt to high level fiber feeding after 4 to 7 days, after which gas production will be reduced to normal levels. In addition, humans may adapt to high level fiber feeding by gradually increasing their fiber consumption over a 7 day period. This generally will circumvent the problem outlined above. Pullulan should be no different than any other fiber in this regard.

In summary, I see no problem with pullulan feeding at levels approximating 25 grams/1,000 kcal. Given its chemical structure, its rate and extent of digestion should be relatively high, it should exert no untoward effects on the microbial ecosystem of the large bowel or on the morphology of the organ itself, and no negative systemic effects should be demonstrated as a result of its consumption.

Sincerely,

[Redacted signature box]

George C. Fahcy, Jr.
Professor
Animal Sciences/Nutritional Sciences

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December 21, 2001

Dr. George Fahey writes the following:

"A number of glucose-based oligosaccharides and polysaccharides exist, some of which may appear in the human diet. These include cellobiose and cellodextrins, beta-cyclodextrins, Fibersol-2 (a resistant maltodextrin), gentiooligosaccharides, glucooligosaccharides, starch, and cellulose. These compounds vary widely in structure and, as a result, affect site (small intestine vs. colon) and extent of digestion. None are known to be harmful to humans, and several have been tested in clinical settings.

Using starch as an example, it is well known that rate of digestion is affected by the relative amounts of amylose and amylopectin in the polysaccharide. Goddard et al. (1984) explained that the rate of amylose digestion is slower than that of amylopectin because it is a linear molecule whose glucose units participate more readily in hydrogen bonding than do those in the more highly branched amylopectin. This tends to make them less accessible to enzymatic digestion (Thorne et al., 1983). Further, amylopectin is a larger molecule with more surface area for enzymatic attack.

Starch structure and its digestibility may be modified purposely or inadvertently as a result of food preparation. Annison and Topping (1994) reported that starch structure was physically modified by retrogradation such that it became partially inaccessible to enzymatic attack, a fraction of starch known today as resistant starch. Nutritionally, starches can be grouped into glycemic starches and resistant starches. The former are starches digested in the small intestine into maltose and glucose by alpha-amylase secreted by the pancreas. These sugars are absorbed through the small intestinal wall into the bloodstream and function as the main source of energy for metabolism. Resistant starches are the sum of starches and products of starch degradation that resist digestion and absorption in the small intestine of healthy humans. These starches pass into the colon where they are fermented by colonic microflora, generating short chain fatty acids (acetate, propionate, butyrate) and gases (carbon dioxide, methane, hydrogen).

Vonk et al. (2000) found that highly digestible (~80%) cornstarch contained 26% amylose and 74% amylopectin whereas resistant cornstarch contained 62% amylose and 38% amylopectin. This and other studies have shown that digestibility of resistant starch can drop to ~50%. Likewise, maltodextrins generally are considered to be highly digestible; however, the addition of heat and enzymatic hydrolysis during preparation of maltodextrins can create a greater variety of bond formations including beta-1,3, beta-1,4, and beta-1,6 linkages. Glucose

000119

digestibility of these modified maltodextrins has been shown to be lower than that of unmodified maltodextrins.

Clinical studies conducted in our laboratory and in the laboratories of others have shown that a range of glucose polymers have been well tolerated by animals and humans, and no adverse health effects have been noted. Pullulan also is a glucose polymer and its linear structure with limited branching appears to reduce its digestibility. There is no indication that pullulan would be less well tolerated than another glucose-based oligosaccharide or polysaccharide mentioned above. Indeed, a safety study performed on a similar carbohydrate, Fibersol-2 (a resistant maltodextrin produced by a combination of hydrolysis and transglucosidation reactions [that occur during hydrolysis]) indicates neither acute toxicity (LD-50 in rats of greater than 20 g/kg) nor mutagenicity (Ohkuma and Wakabayashi, 2001). Long term administration did not affect animal growth, weight of internal organs, or any blood biochemical characteristic. No diarrhea occurred until levels of 1 g/kg body weight was fed."

Ohkuma, K. and S. Wakabayashi. 2001. Fibersol-2: a soluble, non-digestible, starch-derived dietary fiber. pp. 509-523. In: B. V. McCleary and L. Prosky (ed.) Advanced Dietary Fibre Technology. Blackwell Science, Oxford, UK. December 21, 2001

Sincerely,



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000120

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Appendix II
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000121

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Appendix III
Production Process

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Appendix III Production Process

Appendix III. Production Process

1. Production Process Overview

HBC Pullulan is produced by mesophilic (22°-30°C) fermentation of a starch syrup with the black yeast, *Aureobasidium pullulans*. *Aureobasidium pullulans* is non-pathogenic, and strains of the organism which produce the least amount of black-pigment, and which require the shortest cultivation periods are used to yield maximum quantities of Pullulan. Pullulan is elaborated extracellularly into the culture medium from which it is recovered and purified as described below [Catley, 1971 Vol 2 Tab 4].

The taxonomy of *Aureobasidium pullulans* has been detailed by William Bridge Cooke in his 1961 publication covering the black yeasts [Cooke, 1961, Vol 2 Tab 6]. The genus *Aureobasidium* belongs to a group of *Fungi Imperfecta*, in which several spores are produced on a conidiophore or a conidiophore-like structure. The most common species within this genus is *Aureobasidium pullulans*. Cooke described the problems of fungal taxonomic development, but concluded that the genus *Aureobasidium* (formerly known as *Pullularia*) is the type genus for the family *Aureobasidiaceae* [Cooke, 1961, Vol 2 Tab 6].

The black yeasts are common soil fungi, found on decaying plant matter, and on fruit, such as the seeds of grapes, where they are common spoilage organisms [Durrell, 1967, Vol 2 Tab 7]. The dark pigmentation of the yeast is due to melanin, which stains the cell walls, and may be heavily encrusted on older cells [Durrell, 1967, Vol 2 Tab 7]. *Aureobasidium pullulans* has a global distribution, where it acts as a plant pathogen, and an agent of decay [Ajello, 1978 Vol 2 Tab 1]. It is unique as a fungus, in that it plays all three roles ascribed to fungi in the ecosphere: it decomposes dead organic material, it acts as a pathogen to plants, and it acts as a symbiont with other organisms, such as lichens [Zabel, *et al*, 1978 Vol 4 Tab 55]. As such *Aureobasidium pullulans* is ubiquitous in nature. *A. pullulans* is an important saprobe in wood products, causing sap wood stain, and mildew on paint, it is a pathogen on some fruits, and vegetables, and as mentioned above, it is reported to be symbiotic with certain lichens [Zabel, *et al*, 1978 Vol 4 Tab 55].

Appendix III Production Process

Pullulan was described in 1959 by Bender, *et al.*, who reported that the polysaccharide was formed in Czapek-Dox medium, at a 20-22% yield on glucose, saccharose, and fructose. Thiamine was stimulatory to Pullulan production, increasing the yield to 32%. The optical activity in water of the polymerized product resulting from the work of Bender and his colleagues was $[\alpha]_D^{20} + 168^\circ$ [Bender, *et al.*, 1959 Vol 2 Tab 2]. These authors stated that glucose was the sole product of hydrolysis, and that the material was similar to the bacterial dextrans.

In 1971 Catley reported on the carbon sources utilized by what then was called *Pullularia pullulans* [Catley, 1971 Vol 2 Tab 4]. The author stated that after about 100 hours of incubation, the culture gradually, darkened and became black, due to the production of melanin pigment. Catley confirmed through paper chromatography following acid hydrolysis and depolymerization with pullulanase that maltotriose was the major oligosaccharide present, and that glucose was the major monosaccharide [Catley, 1971 Vol 2 Tab 4]. In this study traces of tetrasaccharide and galactose were also found. Table 1 demonstrates the effect of carbon source on the yield of Pullulan.

Appendix III Production Process

Table 1
Effect of Carbon Source on the Yield and Purity of Pullulan¹

| Carbon Source (5%) | Extra-Cellular Polymer (mg/ml) | Percent Poly-saccharide (Range of 3 Methods)² | Percent Pullulan of the Poly-saccharide³ | Percent Maltotriose Content of the Polymer |
|---------------------------|---------------------------------------|---|--|---|
| Sucrose | 14.8 | 72-76 | 89 | 76 |
| Maltose | 4.9 | 53-61 | 46 | 52 |
| Glucose | 8.8 | 65-69 | 65 | 61 |
| Fructose | 6.8 | 56-57 | 60 | 29 |

¹ Grown at 25-27°C for 100 hours.

² Acid hydrolysis followed by either Nelson's Method, Glucose Oxidase, or the Phenol-Sulfuric Acid procedure.

³ Based on the glucose content.

The authors concluded that *Pullularia pullulans* preferred glucose over fructose based on data which showed that sucrose was hydrolyzed nearly completely, before the log phase of growth began [Catley, 1971 Vol 2 Tab 4]. Pullulan elaboration apparently occurs only when the cells are in the late log phase of growth. Interestingly, the polymer continues to be produced even when the cells have reached the stationary phase [Catley, 1971 Vol 2 Tab 4]. The authors stated that the conditions for the production of Pullulan are reminiscent of those in which bacteria accumulate polysaccharide. This tends to occur as a result of limiting growth conditions in the presence of an excess of carbon.

Over the last two decades Hayashibara Company, Ltd. has optimized the production of Pullulan. The general production process and its possible variations will be discussed in the following section.

Appendix III Production Process

2. The Hayashibara Process

The strain of *Aureobasidium pullulans* used for the production of Pullulan is labeled "Hayashibara strain". The organism and the particular strain are non-pathogenic and non-toxicogenic, and are not the product of genetic modification. To assure that a pure culture of the Hayashibara strain is used in Pullulan production, stock cultures are freeze-dried and stored in ampules. At the time of cultivation, stock cells are cultured from the ampules and streaked on agar plates. If, after colony formation, the purity of the culture is confirmed, one colony is transferred to an agar slant. This colony is then used as the inoculum for the production of Pullulan.

To guarantee the purity of the culture, the containers and culture media used for cultivation are thoroughly sterilized and the air, used for aeration of the culture, is filtered. At regular intervals during fermentation, microscopic examination, pH determination of the culture and an analysis of Pullulan yield are conducted to assure purity.

If a culture is found to be contaminated, it is heat sterilized and discarded. The source of the contamination is determined to the extent possible, and appropriate counter measures are adopted to prevent recurrences.

Live organisms of the Hayashibara strain are killed by heat sterilization in the course of producing Pullulan. The absence of the live strain in the product is determined by dissolving Pullulan in sterilized water which is added to an agar plate able to support growth of *A. pullulans*. Colonies are observed macroscopically and microscopically to assess whether they can be identified as Hayashibara strain. This strain exhibits characteristic growth morphology, and is therefore recognizable. For those colonies that are difficult to classify, inoculation into liquid medium and assessment of the colony's ability to produce Pullulan is determined. The black pigment produced by the Hayashibara strain is decolorized with activated carbon following pH adjustment of the culture medium.

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Appendix III Production Process

3. Process Description

| Process | Steps |
|---------------------|--|
| 1. Slant Culture | Prepare the slant culture medium. After sterilization, inoculate a slant with cells or spores and culture for 48 hours at 27°C. |
| 2. Seed Cultures | 1) Triangle Flask Culture Prepare 15 flasks of culture medium. After sterilization, inoculate each flask with cells or spores and culture under agitation for 60 hours at 27°C. 2) First Seed Culture Prepare the first seed culture medium. After sterilization, inoculate a triangle flask and culture for 20 hours at 27°C. 3) Second Seed Culture Prepare the second seed culture medium. After sterilization, use the first seed culture as an inoculum and culture in a triangle flask for 20 hours at 27°C. |
| 3. Main Culture | Prepare main culture medium. After sterilization, use the second seed culture as an inoculum and culture for 90 hours at 27°C. |
| 4. Micro Filtration | Remove the fungal cells in the culture solution with a precoated filter. Heat the cell-free filtrate to 130°C. |
| 5. Decolorization | Absorb colored material by adding activated carbon and a filtering aid to the filtrate. Remove foreign substances via filtration. |

Appendix III Production Process

- | | |
|------------------------------------|--|
| 6. Deionization (twin bed) | Cool decolored filtrate to about 25°C. Remove chlorides, proteins and colored substances in the filtrate with an ion exchange resin. |
| 7. Intermediate Evaporation | Evaporate the deionization filtrate to yield approximately 12% solids. |
| 8. Decolorization | Absorb remaining colored material by adding activated carbon and a filtration aid to the concentrated solution. Remove foreign substances by filtration. |
| 9. Final Filtration | Remove any free activated carbon. |
| 10. Final Evaporation | Evaporate final filtrate to yield a 30% concentrate. |
| 11. Drying | Dry the 30% concentrate with a drum-dryer equipped with a scraper. |
| 12. Pulverization | Pulverize the dried fraction with a crusher and classify with a 1.0 mm diameter screen. Convey dry material to a hopper. |
| 13. Filling, Scaling and Packaging | Fill, scale and package the product, after removing it from hopper. |
| 14. Storage | Store packaged product in the store room until quality control assays are completed. |

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Appendix III Production Process

15. Shipping

Ship products after they have been
cleared by the Quality Control
Department.

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Appendix IV Production Controls

Appendix IV. Process Controls Overview

HBC Pullulan is manufactured at Okayama Plant II. Quality control activities from several sections of the company are jointly responsible for Pullulan quality assurance. The manufacturing sections, which are directly responsible are shown below:

| | |
|-----------------|------------------------------|
| Quality Control | Hayashibara Company, Limited |
| | Okayama Plant II |
| | Quality Control Section |
| | Okayama Plant H |
| | Quality Control Section |

Each section has its own analytical laboratory within the plant, equipped with all the necessary laboratory equipment required for the analytical control of raw materials and finished products. If necessary, the plant also has access to other laboratories within the company and the city of Okayama that are equipped to handle more complicated issues. manufactured, and distributed. Analytical methods are designed to provide longitudinal data and information on the identity, purity, quality, strength, and stability of Pullulan. Feedback from the quality control laboratories to the manufacturing plant is used to adjust critical control points if necessary to maintain the desired properties and characteristics of the product. Such a system ensures that HBC Pullulan is manufactured under current Good Manufacturing Practice (cGMP), and that it will meet its published specifications.

Appendix IV Production Controls

1. Manufacturing Process Controls

The following tables list the process controls that have been put in place by the Hayashibara Company, Ltd. to ensure the quality of HBC Pullulan products. Table 1 summarizes the frequency of the Critical Process Controls and Table 2 provides standards for each control, and the sampling details.

Table 1
Frequency of Critical Process Controls

| Manufacturing Process | Critical Control Point | Frequency of Sampling |
|------------------------------|-------------------------------|------------------------------|
| Erlenmeyer Culture | Viability | End of Incubation |
| First Seed Culture | Viability | End of Incubation |
| Second Seed Culture | Viability | End of Incubation |
| Main Culture | Temperature | Constant |
| | Aeration | Constant |
| | pH | Every 8 hours |
| | Viscosity | Every 8 hours |
| | Oligosaccharides | Every 8 hours |

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Appendix IV Production Controls

Table 1
Frequency of Critical Process Controls (Continued)

| | | |
|------------------------------|---------------------|----------------|
| Microfiltration | pH | Every 8 hours |
| | Viscosity | Every 24 hours |
| Decolorization | pH | Every 8 hours |
| Twin Bed Deionization | pH | Every 8 hours |
| | Specific Resistance | Every 8 hours |
| Microfiltration | pH | Every 8 hours |
| | Viscosity | Every 24 hours |
| Decolorization | pH | Every 8 hours |
| Final Filtration | pH | Every 8 hours |
| | Viscosity | Every 24 hours |
| Drying | pH | Every 24 hours |
| | Viscosity | Every 24 hours |

000137

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Pagination**



Computer Technology Services, Inc.

000138

Appendix IV Production Controls

Table 2
Detail of Manufacturing Process Controls and Sampling Procedures

| Manufacturing Process | Item | Standard | Assay Method | Sampling | |
|-----------------------|-----------|----------|--------------|-----------------------------------|----------|
| | | | | Method | Quantity |
| Erlenmeyer Culture | Viability | >400mg/m | Turbidimeter | Pipette from the Erlenmeyer flask | ~ 100 ml |
| First Seed Culture | Viability | >300mg/m | Turbidimeter | Discharge from Sampling Cock | ~ 100 ml |
| Second Seed Culture | Viability | >400mg/m | Turbidimeter | Discharge from Sampling Cock | ~ 100 ml |

000139
 000139

Appendix IV Production Controls

Table 2 Manufacturing Process Controls (Continued)

| Manufacturing Process | Item | Standard | Assay Method | Sampling | |
|-----------------------|------------------|----------------------------------|------------------|---------------------------------|----------|
| | | | | Method | Quantity |
| Main Culture ml | Temperature | 26 - 28°C | Thermometer | - | - |
| | Aeration | >92.2% | Flow Meter | - | - |
| | pH | 4.70-5.00 | pH Meter | Discharge from Sampling Cock | ~100 |
| | Final Viscosity | 170 - 180. mm ² /s | Viscometer | " | " |
| | Oligosaccharides | 6.0-8.5% | Anthrone-Sulfate | " | " |
| Microfiltration | pH | 7.5-7.7 | pH Meter | " | " |
| | Viscosity | 160 - 175 mm ² /s | Viscometer | " | " |
| Decolorization | pH | 4.9-5.5 | pH Meter | " | " |

000140
000140

Appendix IV Production Controls

Table 2 Manufacturing Process Controls (Continued)

| Manufacturing Process | Item | Standard | Assay Method | Sampling | |
|------------------------------|---------------------|----------------------------|-------------------|------------------------------|----------|
| | | | | Method | Quantity |
| TB ¹ Deionization | pH | 5.5-10.0 | pH Meter | Discharge from Sampling Cock | ~100 ml |
| | Specific Resistance | >20,000 cm | Conductive Sensor | " | " |
| Intermediate Evaporation | pH | 5.5 - 6~5 | pH Meter | " | " |
| | Viscosity | 140-170 mm ² /s | Viscometer | " | " |
| Decolorization | pH | 5.5-6.5 | pH Meter | " | " |
| Final Filtration | pH | 5.5-6.5 | pH Meter | " | " |
| | Viscosity | 140-170 mm ² /s | Viscometer | " | " |

000141

Appendix IV Production Controls

Table 2 Manufacturing Process Controls (Continued)

| Manufacturing Process | Item | Standard | Assay Method | Sampling | |
|-----------------------|-----------|------------------------------|--------------|-------------------|----------|
| | | | | Method | Quantity |
| Drying | pH | 5.5-6.5 | pH Meter | Sample from Dryer | ~ 100g |
| | Viscosity | 140 - 170 mm ² /s | Viscometer | | |

Abbreviations:
TB: Twin Bed
K Tower: Cation Tower
A Tower: Anion Tower

000142

End Submission

000143

AM



Hayashibara International Inc.

8670 Wolff Court, Suite 200
Westminster, CO 80030
303-650-4590 (tel) -- 303-650-9860 (fax)

Facsimile Transmission

To: **Dr. Robert I. Merker**
Consumer Safety Officer
CFSAN, FDA
HFS-517
200 "C" Street S.W.
Washington, D.C. 20204

Date: March 22, 2002
Phone:
Fax: **202-418-3131**
Total pgs. **22**

From: Alan B. Richards Ph.D.
Hayashibara International Inc.
8670 Wolff Ct., Ste. 200
Westminster, CO 80031

Dear Dr. Merker:

Please find enclosed the requested copy of the article by Okada, et al.

Note that the first part is the original article in Japanese with the abstract and tables in English. Following, there is a translation of the text next.

Sincerely,

Alan B. Richards, Ph.D.

If there is a problem with this transmission, please call 303-650-4590

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000148

Pages 000149 - 000169 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

AM



**LEE B. DEXTER & ASSOC.
TECHNOLOGY CONSULTANTS**

**15704 WEBBERVILLE ROAD
AUSTIN, TEXAS 78724 USA**

**TELEPHONE (512) 276-7408
FAX (512) 276-7489**

MEMORANDUM

TO: DR. LINDA KAHL PAGES: 3

CC: DR. ALAN RICHARDS

FROM: LEE B. DEXTER

DATE: MARCH 26, 2002

**RE: GRAS NOTIFICATION FOR HAYASHIBARA PULLULAN
MEMORANDUM OF TELEPHONE CONVERSATION**

000178

LEE B. DEXTER & ASSOC.
TECHNOLOGY CONSULTANTS

15704 WEBBERVILLE ROAD
AUSTIN, TEXAS 78724 USA

TELEPHONE (512) 276-7408
FAX (512) 276-7489

March 21, 2002

Dr. Linda Kahl
Office of Food Additive Safety
Center for Food Safety and Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Dear Dr. Kahl:

GRAS Notification for Hayashibara Pullulan
Memorandum of Telephone Conversation

This letter provides a record of our recent telephone conversation regarding the availability of additional data supporting the safety of Hayashibara Pullulan. During the review of the Pullulan GRAS Report, a member of the Expert Panel asked whether or not *Aureobasidium pullulans*, the source organism for Pullulan, produced an Aureobasidin that might affect the health of humans. Aureobasidins are substances with anti-fungal activity, which are produced by certain strains within the genus *Aureobasidium* (These were discussed in Section II, page 20, # 7 in the Notification). As we discussed, the Pullulan Notification contains an Expert Opinion by two well-known mycologists (Ajello and Ahearn), who indicated that one would not expect *A. pullulans* to produce substances toxic to humans.

Nonetheless, in order to confirm that no Aureobasidins were present in Hayashibara Pullulan, the company performed a fungal inhibition study using two strains of *Saccharomyces cerevisiae* as the test organisms. Three lots of Pullulan culture medium and three lots each of products PF-20 and PI-20 were assayed for their ability to inhibit the growth of *Saccharomyces cerevisiae*. As predicted, no inhibition was detected.

Although you stated that it was not necessary to submit this data as an amendment, it is available upon request. Please call either Dr. Alan Richards at (303) 650-4590 or myself if the need should arise.

000179

Page 2

March 21, 2002
Dr. Linda Kahl

Sincerely,

Lee B. Dexter
Technical Consultant

CC: Alan B. Richards, Ph.D., Vice President and General Manager,
Hayashibara International Inc.
Robert Merker, Ph.D., CFSAN, FDA
Mr. Katsuaki Hayashibara, Director, Overseas Business Development
Hayashibara Company, Ltd.

000180

Reference List for Industry Submission, GRN 000099

| <i>Pages</i> | <i>Author</i> | <i>Title</i> | <i>Publish Date</i> | <i>Source</i> | <i>BIB_Info</i> |
|--------------------|---|---|---------------------|------------------------------|--|
| 000149 - 000169 | K. Okada; Yoneyama, M.; Mandai, T.; Aga, H.; Sakai, S.; Ichikawa, T. | Digestion and Fermentation of Pullulan | 1990 | Jpn. Soc. Nutr. Food Sci. | Volume 43, Number 1, pgs 23 - 19 |

NA- Not applicable
