

United States Department of Agriculture
Agricultural Marketing Service | National Organic Program
Document Cover Sheet

<https://www.ams.usda.gov/rules-regulations/organic/national-list/petitioned>

Document Type:

National List Petition or Petition Update

A petition is a request to amend the USDA National Organic Program's National List of Allowed and Prohibited Substances (National List).

Any person may submit a petition to have a substance evaluated by the National Organic Standards Board (7 CFR 205.607(a)).

Guidelines for submitting a petition are available in the NOP Handbook as NOP 3011, National List Petition Guidelines.

Petitions are posted for the public on the NOP website for Petitioned Substances.

Technical Report

A technical report is developed in response to a petition to amend the National List. Reports are also developed to assist in the review of substances that are already on the National List.

Technical reports are completed by third-party contractors and are available to the public on the NOP website for Petitioned Substances.

Contractor names and dates completed are available in the report.



January 31, 2018

National List Manager
USDA/AMS/NOP, Standards Division
1400 Independence Ave. SW
Room 2648-So., Ag Stop 0268
Washington, DC 20250-0268

RE: Petition to add Pullulan to the National List at §205.605(a) as an allowed nonsynthetic ingredient in tablets and capsules for dietary supplements labeled “made with organic (specified ingredients or food group(s)).”

Dear National List Manager:

The Organic Trade Association¹ is filing the attached petition on behalf of its National List Innovation Working Group to add Pullulan to the National List at §205.605(a) as an allowed non-agricultural, non-synthetic ingredient used in tablets and capsules for dietary supplements labeled “made with organic (specified ingredients or food group(s)).” The purpose of submitting this petition is to protect the continued production and availability of USDA-NOP certified encapsulated dietary supplements and to support the commercial development of certified organic Pullulan.

Pullulan is a natural extracellular polysaccharide excreted by the yeast-like fungus *Aureobasidium pullulans*. It is not genetically modified and it is commercially produced by a non-pathogenic and non-toxicogenic strain of the organism using a liquid starch syrup as the fermentation substrate. Pullulan can be made into very thin films with high tensile strength and stability over a range of temperatures, making it an ideal material to be used in the manufacture of empty capsules for the purpose of encapsulating dietary supplements or as a coating for dietary supplement tablets.

Encapsulated vegetarian dietary supplements certified under USDA’s National Organic Program (NOP) rely on the use of Pullulan as the primary ingredient in the capsule. For dietary supplements, the capsule is considered an “ingredient” and must either be “certified organic,” or comprised of ingredients compliant with the National List of Allowed and Prohibited Substances. Since the early 2000s, accredited certifying agents have classified Pullulan as “**agricultural**” and allowed its use only in encapsulated dietary supplements certified to the “**made with**” product category. This allowance has significantly contributed to the growth of NOP certified dietary supplements. Currently, certified organic Pullulan is commercially unavailable in North America and there are no other NOP compliant vegetarian options available. Gelatin capsules, while allowed under NOP, present consumer acceptance and GMO challenges.

¹ The Organic Trade Association (OTA) is the membership-based business association for organic agriculture and products in North America. OTA is the leading voice for the organic trade in the United States, representing over 9,500 organic businesses across 50 states. Our members include growers, shippers, processors, certifiers, farmers' associations, distributors, importers, exporters, consultants, retailers and others. OTA's mission is to promote and protect organic with a unifying voice that serves and engages its diverse members from farm to marketplace.

In late 2016, NOP released a guidance document (NOP 5033) on the Classification of Materials. This document assists the National Organic Standards Board (NOSB), accredited certifying agents, and the organic industry in making ‘Agricultural’ vs. ‘Non-agricultural’ and ‘Synthetic’ vs. ‘Non-synthetic’ determinations. Given the information contained in the NOP guidance document, accredited certifying agents are now in general agreement that Pullulan should be classified as a “**non-agricultural, non-synthetic**” substance and accordingly must appear on the National List at §205.605 to be allowed in NOP certified products.

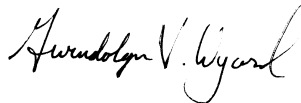
In response to this new interpretation, we are requesting that Pullulan be added to the National List so that it may *continue* to be allowed as an ingredient in capsules for dietary supplements labeled “made with organic (specified ingredients or food group(s)).” Without its continued allowance, and without an alternative option, we estimate the economic impact to the organic dietary supplement sector would be over \$825 million. Please note that we are intentionally limiting the allowance of non-organic Pullulan to dietary supplements certified to the “made with” category. Any encapsulated dietary supplement sold or labeled as “certified organic (95%+) will need to use certified organic Pullulan. Although organic Pullulan based capsules are not commercially available in North America, development is underway and they should be available in the future.

In summary, adding Pullulan to the National List will:

- Quickly address a new interpretation made by several accredited certifying agents in response to NOP’s Classification of Materials Guidance (NOP 5033);
- Prevent widespread disruption and economically significant damage to the organic supplements sector;
- Bring the allowance of non-organic Pullulan under strict review of NOSB and the National List Sunset process;
- Support the commercial development of certified organic Pullulan.

Thank you for your assistance in putting this petition before NOSB. If you need any additional information, please contact me via e-mail or telephone: gwyard@ota.com or (503) 798-3294.

Respectfully submitted,



Gwendolyn Wyard
Vice President, Regulatory and Technical Affairs
Organic Trade Association

cc: Laura Batcha
Executive Director/CEO
Organic Trade Association

Pullulan Petition

1/31/2018

Item A

This petition seeks inclusion of **Pullulan as an ingredient in tablets and capsules for dietary supplements labeled in the “made with organic (specified ingredients or food group(s))” category** on the National List at §205.605(a).

Item B

1. Substance Name.

Common Name: Pullulan

Chemical Name:

Poly[6)- α -D-glucopyranosyl-(1 \rightarrow 4)- α -D-glucopyranosyl-(1 \rightarrow 4)- α -D-glucopyranosyl-(1 \rightarrow]

CAS Number: 9057-02-7

EC / EINECS Number: 232-945-1

Empirical Formula: (C₆H₁₀O₅)_n where n=300 to 3000

Pullulan is a natural extracellular polysaccharide excreted by the yeast-like fungus *Aureobasidium pullulans* (formerly known as *Pullularia pullulans*). It is not genetically modified and it is commercially produced by a non-pathogenic and non-toxigenic strain of the organism using a liquid starch syrup as the fermentation substrate. Pullulan is a linear glucan consisting of repeating units of maltotriose joined by α -D-(1 \rightarrow 6) linkages.

2. Petitioner and Manufacturer Information.

Petitioner: Organic Trade Association
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Contact Person: Gwendolyn Wyard
Title: Vice President of Regulatory & Technical Affairs
Phone: (503) 798-3294
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Manufacturer: Hayashibara Co., Ltd.
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Phone: +81-86-224-4312

3. Intended or Current Use.

Pullulan can be made into very thin films with high tensile strength and stability over a range of temperatures. Pullulan films have a low oxygen permeability, are oil and grease resistant and dissolve rapidly in water. These films may be shaped using rapid evaporation of water, compression molding or extrusion at high temperatures, making it an ideal material to be used in the manufacture of empty capsules for the purpose of encapsulating dietary supplements or as a coating for dietary supplement tablets.

Non-animal capsules and tablets are suitable for a variety of cultural and dietary requirements, including those of vegetarians, diabetics, and people with restricted diets for health or religious reasons.

The danger from animal plagues such as mad cow disease and foot-and-mouth disease raised concerns about raw materials of animal origin being used in foods. Questions surrounding the GMO status of animal feed have also been pervasive in the natural products industry. The requests for raw materials of plant origin for use in foods, cosmetics and the medical fields have been increasing. Pullulan is produced from starch and, therefore, demand for pullulan capsules has grown significantly, as evidenced by the more than 42% growth in sales over the past two years.

Pullulan is also currently used in a wide range of other applications. These include as coatings and binders for pharmaceutical tablets, as a thickener in sauces, jams, jellies and confections, postharvest coatings for fruits and nuts, cosmetics, and oral hygiene products. Because of its biocompatible properties, pullulan is also the base material used in a host of biomedical applications.

4. Intended Activities and Application Rate.

For the purposes of this petition, we will focus on pullulan's primary use in the manufacture of dietary supplements as described above.

Hard capsules are manufactured by dipping stainless steel pins into a pullulan solution (usually 5-10%), then dried. The amount of pullulan in the final capsule is approximately 80% or greater, with water being the next highest constituent. Pullulan coatings and films may be as thin as 0.01 mm.

In the Pullulan GRAS Notification submitted by Hayashibara International Inc. to FDA in 2002 (See Appendix A), it was estimated that the daily intake of pullulan as a capsule would be approximately 0.69g per day.

5. Manufacturing Process.

Hayashibara Company Limited in Okayama, Japan, began commercial production of pullulan in 1976. Pullulan production was a natural outgrowth of Hayashibara's original business of starch syrup production, founded in 1883. Hayashibara adjusted the growth conditions of a specified strain of *Aureobasidium pullulans* to produce pullulan products of particular molecular weights and

specifications. These include food grade and pharmaceutical grade products with mean molecular weight ranging from 100,000 to 200,000. Pullulan films were commercialized by Hayashibara in 1982. Today, this company is still the principal commercial producer of pullulan.

The *A. pullulans* strain that is used is non-toxicogenic and has been selected by traditional techniques, i.e., the strain is not the product of genetic modification using recombinant technologies. The production strain has a high yield of pullulan and low production of black pigment (melanin).

The manufacturing process is conducted under conditions of good manufacturing practices and uses raw materials and processing aids that comply with food grade specifications. Pullulan is produced by mesophilic (22-30°C) fermentation of *A. pullulans* in a suitable starch syrup. During fermentation, pullulan is secreted extracellularly by the organism into the culture medium from which it is then recovered and purified.

At the completion of fermentation, the resulting broth consists of microbial cells and cellular debris, as well as the extracellular metabolites produced and excreted during the fermentation (e.g., pullulan). The microbial cells and cellular debris are first removed by microfiltration. The cell-free filtrate is then heat-sterilized.

The filtrate is then purified by a deionization process using an ion exchange resin to remove the salt and protein contaminants¹. The deionized solution is concentrated to a solids content of about 12%, treated with activated carbon to remove pigments and other impurities by adsorption, and filtered using diatomaceous earth as a filter aid.

The filtrate is concentrated by evaporation to a solids content of about 30% and dried in a drum dryer. The dried pullulan is pulverized to a specified particle size and packed in sterilized polyethylene bags.

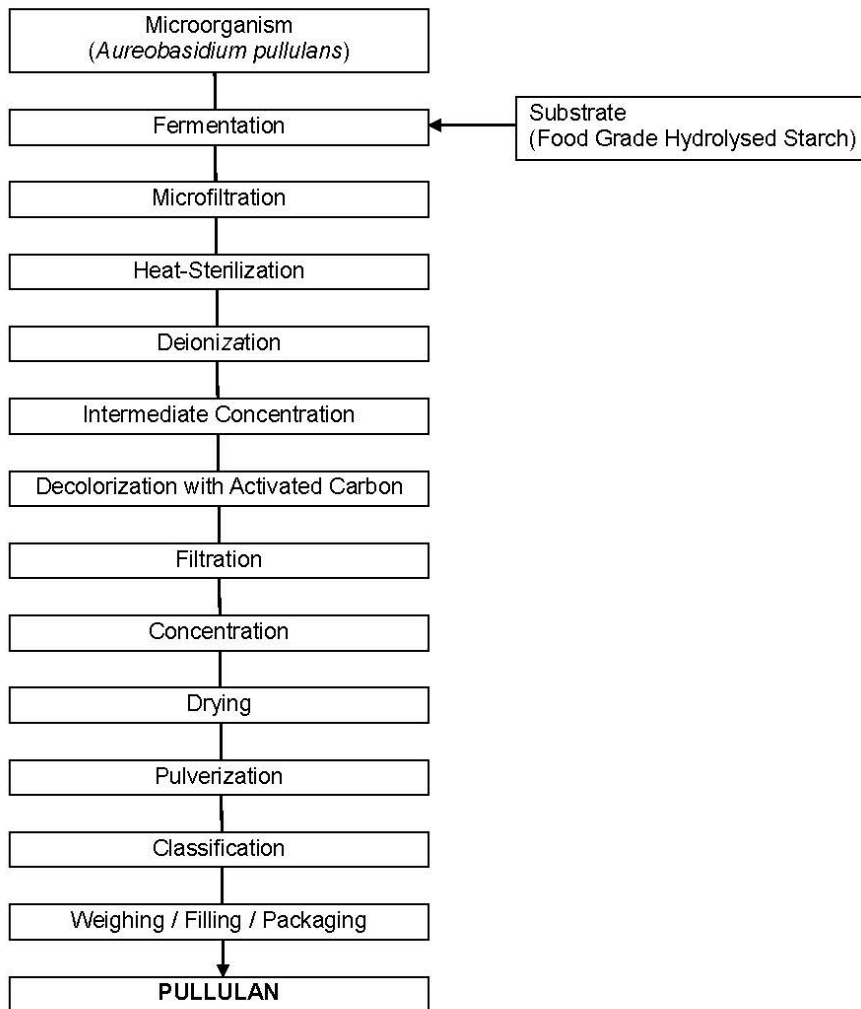
The final pullulan product is tested in accordance with global compendia test methods and specifications as referenced in the Japanese Pharmacopoeia, the European Pharmacopoeia, the United States Pharmacopoeia and the National Formulary, and the Food Chemicals Codex.

¹ Please note that some manufacturing descriptions found in research articles indicate that an additional step may be utilized prior to deionization using ion-exchange chromatography in which the filtrate is treated with an organic solvent (e.g., alcohol) to precipitate the pullulan. However, we have confirmed that Hayashibara Company's manufacturing process does not use this step and no organic solvents are used during their production.

January 29, 2018

To: Capsugel

Subject: JP PULLULAN LMO / Manufacturing Flow Chart



Makoto Kikkawa

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6. Ancillary Substances.

There are no ancillary substances such as carriers, emulsifiers or stabilizers in the final pullulan product.

7. Previous Reviews.

National Organic Standards Board

In 2004, a petition to add pullulan to §205.605 of the National List was submitted to the National Organic Standards Board by Capsugel Division of Pfizer Inc. NOSB subsequently put this petition on hold and no final recommendation for this substance was ever made. At this time, it is unclear why the petition was put on hold as we could not find any references to it in the NOSB meeting minutes, and Handling Subcommittee meeting notes for this time period are not available on the USDA NOP website.

However, prior to the finalization of Guidance document *NOP 5033—Classification of Materials*, non-organic pullulan was commonly classified as an agricultural ingredient and was therefore allowed in products labeled “made with organic.” Due to this interpretation, many organic supplements were developed to use non-organic pullulan capsules and currently are being marketed in the “made with organic” category.

After NOP 5033 was finalized, some certifiers have reclassified pullulan as a non-agricultural ingredient based upon the decision tree published in NOP 5033-2. This has created a challenge for both certifiers and manufacturers as there is not a clear consensus regarding the classification of this material and whether existing products using it should continue to be allowed in the “made with organic” category.

Recently, the Accredited Certifiers Association created a work group to address this topic. While not all in the industry agree that pullulan should be considered non-agricultural, this work group strongly agreed on this classification. As such, they have suggested that since the National List of Allowed and Prohibited Substances currently does not provide an allowance for pullulan at §205.605, steps should be taken to phase out existing formulations containing this material. However, they have agreed that with the submission of this petition, certifiers should suspend the phase-out efforts until such time that the NOSB reaches a decision about whether pullulan should be added to the National List.

The 2018 forecast for pullulan capsules, the only capsule that is currently allowed in the “made with organic” category, is approximately 2.5 billion capsules. A conservative estimate of \$10 per bottle of 30 would represent an economic value of over \$825 million. The addition of pullulan at §205.605 will be **critical** to maintain the status of these products and avoid consumer confusion. Notably, there is no other alternative for a vegetarian, organic-compliant capsule and companies would be forced to either lose organic certification without changing their formula, or switch to a gelatin capsule.

World Health Organization

In the *WHO Food Additives Series: 56 – Safety evaluation of certain food additives* prepared by the 65th meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), the Committee concluded

that the use of pullulan as a food additive and the studies provided to the Committee on its safety provided sufficient information to allocate an Acceptable Daily Intake (ADI) 'not specified'² (See Appendix B).

European Food Safety Authority

In the European Food Safety Authority's (EFSA) *Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food on a request from the Commission related to Pullulan PI-20 for use as a new food additive* (EFSA-Q-2003-138), adopted 13 July 2004 (See Appendix C), the following conclusion was made:

“...On the basis that pullulan is similar to other poorly digested carbohydrates and that the current proposed usage levels are below the level likely to cause abdominal fullness, the Panel consider that the expected intakes of pullulan would not present any concern when used as a food additive in the proposed uses and at the usage levels requested...”

National Industrial Chemicals Notification and Assessment Scheme

In Australia, the National Industrial Chemicals Notification and Assessment Scheme (NICNAS) is administered by the Department of Health and Ageing, and conducts the risk assessment for public health and occupational health and safety. The assessment of environmental risk is conducted by the Department of the Environment and Water Resources. Hayashibara International applied to NICNAS for pullulan so that it could be used in the manufacture of cosmetic products such as shampoos, creams and lotions, styling products (as an impermeable, antistatic solid film) and toothpastes (See Appendix D). In the May 2007 Full Public Report on Pullulan prepared by NICNAS, the following conclusions were made:

8.1 Level of Concern for Occupational Health and Safety

There is Low Concern to occupational health and safety under the conditions of the occupational settings described.

8.2 Level of Concern for Public Health

There is No Significant Concern to public health when used in the proposed manner.

8.3 Level of Concern for the Environment

The polymer is not considered to pose a risk to the environment based on its reported use pattern.

² **ADI 'not specified'** is used to refer to a food substance of very low toxicity which, on the basis of the available data (chemical, biochemical, toxicological and other) and the total dietary intake of the substance arising from its use at the levels necessary to achieve the desired effects and from its acceptable background levels in food, does not, in the opinion of the Committee, represent a hazard to health. For that reason, and for the reasons stated in the individual evaluations, the establishment of an ADI express in numerical form is not deemed necessary. An additive that meets this criterion must be used within the bounds of good manufacturing practice, i.e., it should be technologically efficacious and should be used at the lowest level necessary to achieve its effect, it should not conceal food of inferior quality or adulterated food, and it should not create a nutritional imbalance.

8. Regulatory Authority.

In the USA, the FDA received the generally recognized as safe (GRAS) notification of pullulan in March 2002. The Agency Response Letter issued in August 2002 noted that FDA had no question about the GRAS status of pullulan and designated it as GRAS in Notice GRN 000099 (See Appendix A).

In the U.S. EPA Toxic Substances Control Act (TSCA) Chemical Substance Inventory, the entry for pullulan notes that it is exempt from reporting.

In Japan, pullulan was approved as food ingredient and additive with no limitation of use in Serial #373, April 16, 1999. It appears in the List of Existing Food Additives published on 16 April 1996 (Ministry of Health and Welfare Notification No. 120). The specifications are described in the Specifications and Standards for Foods, Food Additives, etc. (Ministry of Health and Welfare Notification No. 370 issued in 1959 revised on 30 March 2006) and listed in Japan's Specifications and Standards of Food Additives, 8th edition on 30 August 2006. It is listed in the Japanese Standards of Quasi-Drug Ingredients 2006 (Notification No. 0331030 issued by the Director of the Pharmaceutical and Food Safety Bureau, the Ministry of Health, Labour and Welfare). Pullulan and Pullulan Capsules are also listed in the Japanese Pharmacopoeia, 17th edition, published on April 1, 2016.

In the EU, pullulan has the European Community (EC) number 232-945-1 and the E number E1204. Commission Regulation No. EU 231/2012 outlines specifications for pullulan as a food additive for use in capsules, tablets and films under directive 2006/52/EC.

In addition, pullulan is permitted in Russia, China, Korea, Taiwan, Thailand, Singapore, Vietnam and Mercosur of South America (Argentina, Brazil, Paraguay and Uruguay) (Chaen, 2010).

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated the safety of pullulan at the 65th meeting (See Appendix B) and announced the acceptable daily intake (ADI) as 'not specified'. The International Numbering System (INS) number 1204 was assigned to pullulan.

Additionally, pullulan is approved for use in Halal and Kosher foods.

9. Chemical Abstracts Service (CAS) Number and Product Labels.

CAS Number: 9057-02-7

INS Number: 1204

See Appendix E for Specification Sheet.

10. Physical and Chemical Properties.

Pullulan is a white to slightly yellowish powder that is tasteless and odorless. It is non-toxic, non-mutagenic, non-carcinogenic and non-hygroscopic. It has physical properties that are useful in a wide

variety of applications, most importantly in food and nutritional supplements. Its film-forming properties are excellent for creating hard capsules and tablet coatings for supplements. The oxygen resistance of pullulan films is suitable for the protection of readily oxidized fats and vitamins contained within the capsules. Pullulan also provides a better odor barrier of pungent ingredients or compounds versus other capsule types. Pullulan films also inhibit fungal growth.

When dissolved in water, pullulan is highly adhesive and has remarkable binding properties, making it useful for binding and agglomerating powders. Pullulan is capable of being compressed into tablets that release a suspended or solubilized substance over time.

Pullulan is easily dissolved in hot or cold water to form a stable, viscous solution that does not gel. It is not hygroscopic but can retain 10-15% moisture content without becoming sticky. It is stable over a broad range of pH conditions when in solution. It is also relatively stable to heat.

(a) Chemical interactions with other substances, especially substances used in organic production:

Because it does not contain any chemically reactive groups, pullulan is not expected to interact chemically with other substances.

(b) Toxicity and environmental persistence:

Aureobasidium pullulans, the organism that produces pullulan, is a common mold that is ubiquitous in nature. It is widely spread in all ecological niches including forest soil, fresh and sea water, plant and animal tissues, etc. (Cheng *et al.*, 2011). It is non-pathogenic and non-toxic.

Pullulan itself is also non-toxic. It is a fully biodegradable polysaccharide and does not persist in the environment.

In 2005, Dr. Timothy D. Leathers³ addressed how some enzymes produced by various microorganisms break down pullulan:

Pullulan is attacked by glucoamylases from numerous fungi, including species of *Aspergillus*, *Candida*, *Rhizopus*, and *Sclerotium* (Ueda *et al.*, 1963; Wallenfels *et al.*, 1965; Marshall, 1975; Saha *et al.*, 1979; McCleary and Anderson, 1980; De Mot *et al.*, 1985; Kelkar and Deshpande, 1993). Glucoamylases are progressive exo-acting enzymes that attack from the non-reducing end to produce glucose. *Aureobasidium* also has been reported to produce glucoamylases, and it has been postulated that these enzymes may be involved in pullulan degradation in late cultures (Saha *et al.*, 1993; West and Strohfus, 1996a).

³ Leathers, T. D. 2005. Pullulan. Polysaccharides. Biopolymers Online. 6. (Published online 15 January 2005)

Bender and Wallenfels (1961) first described a pullulanase (pullulan 6-glucanohydrolase, EC 3.2.1.41) from *Klebsiella planticola* (also *K. pneumoniae*, and formerly *Aerobacter aerogenes* or *Enterobacter aerobenes*) with specificity for the internal α -(1→6) linkages of pullulan. This enzyme converts pullulan almost quantitatively to maltotriose, which is readily broken down by amylases (Catley, 1978). Because pullulanases also recognize α -(1→6) branch linkages in amylopectin, they sometimes have been referred to as “bacterial isoamylases”. However, isoamylases (EC 3.2.1.68) lacking pullulanase activity are widely produced by many organisms, including bacteria, fungi, plants, and animals (Manners, 1971). Many plants produce both isoamylases and pullulanases as separate activities.

(c) Environmental impacts from its use and/or manufacture:

There are no known negative environmental impacts resulting from the use or disposal of pullulan. It is a biodegradable polysaccharide that is easily metabolized by many microorganisms found in nature to give carbon dioxide and water.

Because of its unique qualities, pullulan may be used as a base material in novel flocculants that have been developed for the removal of contaminants in wastewaters (Ghimici & Constantin, 2011) resulting in a beneficial impact on aquatic environments.

(d) Effects on human health:

Pullulan provides few calories and acts as a soluble dietary fiber. *In vivo* and *in vitro* metabolism and digestion studies in rats and humans demonstrated that pullulan is minimally hydrolyzed by salivary amylase and pancreatic amylase without glucose formation (Oku *et al.*, 1979; Okada *et al.* 1990; Yoneyama *et al.*, 1990). Enzymes of the small intestine also hydrolyze pullulan producing minimal amounts of glucose. A large proportion of the dietary pullulan reaches the large intestine and functions as a prebiotic, selectively promoting the growth of beneficial bifidobacterial (Mitsubishi *et al.*, 1990; Sugawa-Katayama *et al.*, 1994; Yoneyama *et al.*, 1990). Wolf *et al.* (2003) reported that a food grade pullulan is slowly digested in humans, and that half of administered pullulan is absorbed as blood sugar.

Pullulan has been used in Japan in various forms for more than 30 years without reported adverse effects. In addition, pullulan intakes of 10 g/day were well tolerated by human volunteers taking part in a 14-day metabolism study (Yoneyama *et al.*, 1990). The only complaint, which was reported by a few of the participants, was post-intake abdominal fullness. There were no significant changes in the blood biochemistry parameters of the volunteers fed pullulan.

In an oral lethality study, pullulan administered at doses of up to approximately 15 g/kg body weight did not cause any mortalities in mice (Kimoto *et al.*, 1997). The yeast from which pullulan is obtained, *Aureobasidium pullulans*, also was demonstrated to be relatively innocuous, as indicated by oral LD₅₀ values of >24 and >40 g/kg body weight in male adult mice, and male and female Sprague-Dawley rats, respectively (Kimoto *et al.*, 1997).

Results of longer-term repeated dose studies also demonstrated pullulan to be of low oral toxicity (Oku *et al.*, 1979; Kimoto *et al.*, 1997). No toxicologically significant effects were observed in rats fed diets containing as much as 40% pullulan for 9 weeks. Observations of decreased body weight and digestive tract hypertrophy in rats fed high pullulan diets (20-40%) can be attributed to the effect of replacing the normal nutrient content of food with indigestible carbohydrate (LRSO, 1975).

The NO(A)EL, or no observed adverse effect level, for pullulan, based on a 63-week dietary study in rats, was estimated to be 5,000 mg/kg body weight/day (Kimoto *et al.*, 1997). The only treatment-related change noted was an increase in cercal weight in female rats receiving 5,080 mg/kg body weight/day of pullulan (about 10% of diet).

Increased cercal weight is a common physiological response to consumption of poorly digested polysaccharides (El-Harith *et al.*, 1976; Oku *et al.*, 1979; MacKenzie *et al.*, 1986; Olivier *et al.*, 1991).

Mutagenicity studies in strains of *Salmonella typhimurium* and *Escherichia coli*, using the plate incorporation method, demonstrated that pullulan was not mutagenic either with or without metabolic activation (Kuroda *et al.*, 1985; Kimoto *et al.*, 1997). Pullulan also was not found to be clastogenic in a chromosome aberration assay in Chinese hamster lung cells (Ishidate *et al.*, 1985).

In November 2016, the FDA Office of Nutrition and Food Labeling Center for Food Safety and Applied Nutrition published the [Science Review of Isolated and Synthetic Non-Digestible Carbohydrates](#). This document provided a summary of its review of the scientific evidence that it identified for certain isolated or synthetic non-digestible carbohydrates that are not listed as a dietary fiber in 21 CFR 101.9(c)(6)(i). Following is the entry for Pullulan from this document:

Background

Pullulan is a naturally occurring exopolysaccharide produced by Aureobasidium pullulans, a ubiquitous fungus. Pullulan is a glucan consisting predominantly of repeating maltotriose units, which consist of three 1,4-linked glucose molecules, linked by α -1,6-glycosidic bonds (Catley, 1971). Occasionally, maltotetraose units consisting of four 1,4-linked glucose molecules are distributed randomly in the polymer (Catley, 1986). Pullulan is a soluble fiber with film-forming properties that is used as a matrix to hold flavors (breath fresheners), as a coating for foods to extend shelf-life, and as a substitute for gelatin in capsules used for dietary supplements (FDA, 2002).

Blood Cholesterol Levels

*We identified one study that evaluated the effect of pullulan consumption on blood cholesterol levels (Stewart *et al.*, 2010). Scientific conclusions could not be drawn from this study because it was not conducted long enough to evaluate the effect of pullulan on fasting blood cholesterol levels.*

Blood Glucose Levels

We identified five studies that evaluated the effect of pullulan consumption on blood glucose levels. Scientific conclusions could not be drawn from one of these studies (Klosterbuer et al., 2012) because a mixture of non-digestible carbohydrate, including pullulan, was used in the study and the physiological effect of pullulan per se therefore could not be evaluated.

Wolf et al. (2003)

Twenty-eight non-diabetic healthy U.S. adults consumed 50 g of pullulan or maltodextrin (control) in a randomized, double-blinded, cross-over study in which subjects participated in two separate three-hour meal tolerance tests. The incremental peak blood glucose concentration was reduced by 54% when subjects consumed pullulan compared to the control group (4.24 ± 0.35 vs. 1.97 ± 0.10 mmol/L) ($P < 0.001$). At 180 minutes, the blood glucose concentration was higher when subjects consumed pullulan, supporting the hypothesis that pullulan is digested slowly ($P < 0.05$). The positive incremental area under the curve was significantly reduced by 50% when subjects consumed pullulan compared with the control ($P < 0.001$).

Kendall et al. (2008)

Twelve healthy Canadian volunteers participated in a randomized, cross-over study in which they consumed 25 g of glucose in a beverage (control) and seven test beverages that contained 25 g of total carbohydrates. One of the test beverages contained pullulan as the carbohydrate. The measured blood glucose (as well as insulin) area under the curve ($\text{mmol} \times \text{min/L}$) was significantly lower for the study subjects who consumed the pullulan test beverage (8.7 ± 4.1) compared to the control group (103.7 ± 13.7) ($P < 0.05$).

Peters et al. (2011)

Thirty-five healthy subjects from the Netherlands participated in a randomized, double-blind, cross-over study in which they were provided with a test beverage containing 15 g of long-chain pullulan (LCP), medium-chain pullulan (MCP), or maltodextrin (control). Blood samples were collected from only a subset of the study subjects ($n=12$). The blood glucose area under the curve for the period of 0 to 150 minutes was significantly higher for LCP and MCP groups compared to the control group ($P < 0.05$).

Laxation/Bowel Function

We identified one study that evaluated the effect of pullulan consumption on laxation.

Stewart et al. (2010)

In a single-blind, cross-over study, 20 healthy US subjects consumed 12 g/day of one of five non-digestible carbohydrates including pullulan, or maltodextrin (control) for 14 days. There was no significant difference in the number of recorded stools per day between the pullulan and control diets ($P > 0.05$).

Energy Intake

We identified one study that evaluated the effect of pullulan consumption on energy intake (Klosterbuer et al. 2013). Scientific conclusions could not be drawn from this study because a mixture of non-digestible carbohydrate along with pullulan was provided to the study subjects and the physiological effect of pullulan per se therefore could not be evaluated.

To summarize, no adverse effects of toxicological significance have been observed for pullulan in a variety of assays. Pullulan is structurally similar to starch and would not be expected (based on estimated consumption data) to introduce a substantial increase in the level of alpha-1,6 linked glucose, a minor constituent of normal starches, into the diet. Lastly, the safety of pullulan is supported by over 30 years of Human consumption in Japan and by the absence of adverse events in human trials at doses of 10 g pullulan/day to evaluate metabolism and digestion.

(e) Effects on soil organisms, crops, or livestock:

Pullulan is a biodegradable polysaccharide that is easily metabolized by many microorganisms naturally found in soil to give carbon dioxide and water.

While pullulan is not expected to be used in crop production, it has been used as an ingredient in post-harvest coating of fruits and vegetables to extend shelf life and maintain quality.

Feed supplements for livestock would need to be certified organic, therefore, pullulan capsules would not qualify for this use. However, as a non-synthetic material, it could in theory be used for livestock health care. To the best of our knowledge, there are currently no livestock health care products in the organic market that use pullulan, but if there were, we would expect the pullulan to act as a soluble dietary fiber as it does in humans.

11. Safety Information.

Pullulan is not expected to be hazardous when used under normal conditions. However, because it is a powder, appropriate personal protection should be used when it is being handled to avoid respiratory or eye irritation.

See Appendix F for Safety Data Sheet

No substance reports for pullulan were found in the National Institute of Environmental Health Studies or National Toxicology Program databases.

No Health Hazard Evaluation (HHE) reports for pullulan were found in the National Institute for Occupational Safety and Health (NIOSH) database.

12. Research Information.

Pullulan has a long history and due to its unique properties, it has been studied for many different applications. In addition to the research information presented in section 10 above that is relevant to food and dietary supplement uses, there is considerable research for its use as a base for:

- Post-harvest coatings for fruits and vegetables to preserve food quality
- Blood plasma substitute / expander
- Skin, bone, cartilage and smooth muscle cell culture
- Drug and vaccine delivery
- Wound healing
- Post-operative tissue adhesion prevention
- Biodegradable and anti-fog food packaging
- Flocculants for water treatment

See the Supplemental Bibliography included in Appendix G for additional examples of literature regarding uses for pullulan other than as an ingredient in dietary supplements as further evidence of its history of safe use as a food additive, as a biocompatible base material for medical applications, and other purposes.

13. Petition Justification Statement

Encapsulated vegetarian dietary supplement products certified under USDA's National Organic Program (NOP) rely on the use of Pullulan as the primary ingredient in the capsule. For dietary supplements, the capsule is considered an "ingredient" and must either be "certified organic," or comprised of ingredients compliant with the National List of Allowed and Prohibited Substances. Encapsulation of organic raw materials and active blends is an essential to the handling of dietary supplements because it allows for the delivery of materials without the use of excipients, and without the risk of damaging those materials through tablet compression. It also allows for controlled dosage, which bulk powders do not and the lack of heat used during processing helps preserve the bioavailability of the active compounds.

Since the early 2000s, accredited certifying agents have classified Pullulan as "**agricultural**" and allowed its use only in encapsulated dietary supplements certified to the "**made with**" **product category**. This allowance has significantly contributed to the development of NOP certified encapsulated dietary supplements and the growth of the \$1.2 billion organic dietary supplement sector as a whole.

In late 2016, NOP released a guidance document (NOP 5033) on the Classification of Materials. This document assists the National Organic Standards Board (NOSB), accredited certifying agents, and the organic industry in making 'Agricultural' vs. 'Non-agricultural' and 'Synthetic' vs. 'Non-synthetic' determinations. Given the information contained in the NOP guidance document, accredited certifying agents are now in general agreement that Pullulan should be classified as a "**non-agricultural, non-**

synthetic” substance and accordingly must appear on the National List at §205.605 to be allowed in NOP certified products.

In response to this new interpretation, the Organic Trade Association is requesting that Pullulan be added to the National List so that it may *continue* to be allowed as an essential ingredient in capsules for dietary supplements labeled “made with organic (specified ingredients or food group(s)).” Continued allowance is necessary because organic Pullulan is commercially unavailable in North America and there are no other NOP compliant vegetarian options available. We estimate the economic impact to the organic dietary supplement sector would be more than \$825 million should Pullulan be no longer allowed.

Non-synthetic/synthetic substances on the National List, or alternative cultural method that could be used in place of the petitioned synthetic substance:

Pullulan is non-synthetic

As stated earlier, Pullulan is the only non-synthetic NOP compliant option available for encapsulated vegetarian dietary supplements certified under USDA’s National Organic Program. It also meets the requirements for Kosher and Halal. Pullulan is a natural extracellular polysaccharide excreted by the yeast-like fungus *Aureobasidium pullulans*. It is produced through a naturally occurring biological process (fermentation) and does not undergo a chemical change at any stage of the extraction or purification process. The purified substance is non-synthetic. Furthermore, the microorganism is not genetically modified and there are no ancillary substances added.

Organic alternatives

At this time, organic Pullulan based capsules are not commercially available in North America. A quick Internet search for organic Pullulan will produce results reflecting the availability from Bright Pharma Caps, Inc., JC Bright. However, Capsugel® is the owner of US patents covering Pullulan capsules. Capsugel® sued JC Bright for patent infringement and false advertising related to JC Bright’s sale of Pullulan capsules. Capsugel® obtained a consent judgment barring JC Bright from selling infringing organic and non-organic capsules. At this time, we are not aware of a legitimate source of Pullulan capsules in the US other than Capsugel®

Due to the fact that Pullulan is made via a fermentation utilizing agricultural source material, the manufacturing of **organic** Pullulan is possible and development is underway. Capsugel® is in the process of ramping up scale to meet the demand and the availability of organic Pullulan for the US market should occur in the future. However, in the interim, no other vegetarian option is available.

National List alternatives

Gelatin-based capsules, informally called gel caps or gelcaps, are composed of gelatin **manufactured** from the collagen of animal skin or bone. Gelatin is listed on §205.606 of the National List and may be used as an ingredient in gelatin capsules for dietary supplements provided they are non-GMO and not available in organic form. However, gelatin capsules are animal based and not appropriate for vegetarian products and may cause issues among kosher and halal consumers.

Hydroxypropyl methylcellulose (HPMC) based capsules are commonly used as an alternative to gelatin capsules and provide consumers with a vegetarian option. However, HPMC was petitioned to the National List in September 2002 as an ingredient of hard capsules but the recommendation to add the substance failed. NOSB determined that HPMC is manufactured from purified cellulose that they classified as synthetic and the majority concluded that its use is not compatible with organic production.

Appendices

- A. U.S. Food and Drug Administration (FDA) Agency Response Letter GRAS Notice No. GRN 00099 and Original GRAS Notification Submission by Hayashibara
- B. Chemical and Technical Assessment and excerpt from WHO Food Additives Series 56 – Safety evaluation of certain food additives prepared by the 65th meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), Geneva, 2006
- C. The European Food Safety Authority (EFSA) Journal (2004) 85, 1-32: Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids, and Materials in Contact with Food on a request from the Commission related to Pullulan PI-20 for use as a new food additive, Question Number EFSA-Q-2003-138
- D. National Industrial Chemicals Notification and Assessment Scheme (NICNAS) Full Public Report - Pullulan
- E. Hayashibara – Nagase Group Pullulan Specifications
- F. Safety Data Sheet
- G. Bibliography and Supplemental Bibliography
- H. Packaging Information Received with Pullulan from Hayashibara Co., Ltd.
- I. Example of Pullulan Capsules, Packaged for Shipment
- J. Plantcaps™ Capsules Brochure

U. S. Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
August 1, 2002

Agency Response Letter GRAS Notice No. GRN 000099

Dr. Alan Richards
Hayashibara International, Inc.
8670 Wolff Court
Suite 200
Westminster, CO 80031-6953

Re: GRAS Notice No. GRN 000099

Dear Dr. Richards:

The Food and Drug Administration (FDA) is responding to the notice, dated February 13, 2002, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on March 1, 2002, filed it on March 1, 2002, and designated it as GRAS Notice No. GRN 000099.

The subject of the notice is pullulan from *Aureobasidium pullulans*. The notice informs FDA of the view of Hayashibara International, Inc. (Hayashibara) that pullulan is GRAS, through scientific procedures, for use in food in general, including meat products, for multiple technical effects.

As part of its notice, Hayashibara includes the conclusions and signed opinion of a panel of individuals (Hayashibara's GRAS panel) who evaluated the data and information that are the basis for Hayashibara's GRAS determination. Hayashibara considers the members of its GRAS panel to be qualified by scientific training and experience to evaluate the safety of substances added to food.

Hayashibara describes generally available information about the identity, characteristic properties, and functionality of pullulan. Pullulan (CAS Reg. No. 9057-02-7) is an extracellular polysaccharide excreted by the fungus *A. pullulans*. It is an alpha-D-glucan consisting predominantly of repeating maltotrioses linked by alpha-1,6-glycosidic bonds. This repeating sequence forms a stair-step-type structure. Occasional maltotetrose units are distributed randomly throughout the polymer. Molecular weights for pullulan range from 8,000 to 2,000,000 daltons depending on the growth conditions of the organism. Hayashibara adjusts the growth conditions of the source fungus to produce pullulan products of particular molecular weights and specifications. These include food grade (designated as PF) and deionized (PI) products with mean molecular weights of 100,000 (PI-10 and PF-10) or 200,000 (PI-20 and PF-20). Pullulan is soluble in hot and cold water and is generally insoluble in organic solvents. Pullulan is non-hygroscopic and non-reducing; it decomposes at 250 to 280 degrees C. Water solutions are stable, viscous, and do not form gels. The viscosity of water solutions of pullulan is proportional to the molecular weight of the pullulan. Pullulan readily forms a film, which is thermally stable, anti-static,

and elastic. Pullulan has adhesive properties and is directly compressible under heat with moisture.

Hayashibara describes its methods for production of pullulan. The manufacturing process is conducted under current good manufacturing practices and uses raw materials that comply with food grade specifications. Pullulan is produced during mesophilic fermentation of starch syrup by the fungus *A. pullulans*. The culture is micro-filtered to remove fungal cells. The cell-free filtrate is heat sterilized and the absence of culturable *A. pullulans* is confirmed. Filtrates are then decolorized and filtered, yielding a filtrate free of foreign substances. The decolorized filtrate is cooled and deionized with an ion-exchange resin to remove chlorides, proteins and colored substances. The deionized filtrate is evaporated to yield approximately 12 percent solids, then decolorized and filtered again. This filtrate is evaporated to yield 30 percent concentrate, which is dried, pulverized, and classified with a 1.0 mm diameter screen. Hayashibara provides individual specifications for the two food grade pullulan products (HBC Pullulan PF-20 and PF-10), including a specification for lead content of less than 0.1 milligrams/kilogram.

Hayashibara describes pullulan as closely related to amylopectin, dextrin and maltodextrin, and notes that FDA has affirmed the GRAS status of dextrin (21 CFR 184.1277) and maltodextrin (21 CFR 184.1444) for several uses. Hayashibara notes differences between pullulan and these polyglucoses in the relative proportions of alpha-1,4 and alpha-1,6 bonds, the tertiary structure of the molecule, and the extent and mechanism of degradation in the human gut.

Hayashibara describes published information about the safety of the production microorganism, the fungus *A. pullulans*. The information they cite describes *A. pullulans* as ubiquitous in nature, nontoxic and nonpathogenic, and characterizes reports of adverse events associated with *A. pullulans* as extremely rare, restricted to immunocompromised and other high risk individuals, or due to misidentification of the organism.

Using data derived from pullulan consumption in Japan⁽¹⁾, Hayashibara estimates that the daily intake of pullulan from its general use in food is 9.4 grams per person per day (g/p/d) at the mean and 18.8 g/p/d at the 90th percentile⁽²⁾. Hayashibara considers that intake of pullulan is self-limiting due to its organoleptic properties.

Hayashibara presents published and unpublished information related to the safety of pullulan. Hayashibara discusses the fate of pullulan in the digestive tract, referring to a published study using digestive enzymes *in vitro* and fecal culture digestion experiments. Based on this study, Hayashibara concludes that salivary enzymes and enzymes in the upper gastrointestinal tract hydrolyze pullulan only to a limited extent. Hayashibara also concludes that bacteria typical of the distal intestinal tract in humans hydrolyze the pullulan further and ferment the hydrolysis products to short chain fatty acids. Hayashibara also describes a published human consumption study that reported no symptoms other than abdominal fullness; analysis of stool samples from test subjects corroborated that colonic bacteria can hydrolyze pullulan completely and ferment the hydrolysis products. Hayashibara cites a published study that concluded that pullulan was not mutagenic in a bacterial system. Hayashibara describes one published chronic study in rats and three unpublished acute toxicological studies (two in mice and one in rats) and reports that none of these studies showed deleterious effects attributable to the consumption of pullulan.

Based on the information provided by Hayashibara, as well as other information available to FDA, the agency has no questions at this time regarding Hayashibara's conclusion that pullulan from *A. pullulans* is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of pullulan. As always, it is the continuing responsibility of Hayashibara to ensure that food ingredients that the firm markets are safe, and are

otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in your notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the homepage of the Office of Food Additive Safety (on the Internet at <http://www.cfsan.fda.gov/~lrd/foodadd.html>).

Potential labeling issues

Under section 403(a) of the Federal Food, Drug, and Cosmetic Act (FFDCA), a food is misbranded if its labeling is false or misleading in any particular. Section 403(r) of the FFDCA lays out the statutory framework for a health claim. In describing the intended use of pullulan and in describing the information that Hayashibara relies on to conclude that pullulan is GRAS under the conditions of its intended use, Hayashibara raises a potential labeling issue under these labeling provisions of the FFDCA. This labeling issue consists of the description of pullulan as "soluble" fiber. If products that contain pullulan bear any claims about such benefits on the label or in labeling, such claims are the purview of the Office of Nutritional Products, Labeling, and Dietary Supplements (ONPLDS) in the Center for Food Safety and Applied Nutrition (CFSAN). The Office of Food Additive Safety (OFAS) neither consulted with ONPLDS on this labeling issue nor evaluated the information in your notice to determine whether it would support any claims made about pullulan on the label or in labeling.

Use in meat products

During its evaluation of GRN 000099, FDA consulted with the Labeling and Consumer Protection Staff of the Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA). Under the Federal Meat Inspection Act and the Poultry Products Inspection Act, FSIS is responsible for determining the efficacy and suitability of food ingredients and additives in meat and poultry products as well as prescribing safe conditions of use. Suitability relates to the effectiveness of the ingredient in performing the intended purpose of use and the assurance that the conditions of use will not result in an adulterated product, or one that misleads consumers.

FSIS advised that Hayashibara did not provide data to support the use of pullulan as suitable for use in meat products. FSIS states that it cannot consider Hayashibara's notice complete until Hayashibara provides data to FSIS that establish that the ingredients are being used at the lowest level necessary to achieve the intended technical effects in the specific meat products (i.e., product category/type) to which application is desired.

The Federal meat inspection regulations list specific binding additives for use below 3.5 percent of meat product formulation. FSIS has viewed the use of binders and extenders at levels greater than 3.5 percent as re-characterizing products. If Hayashibara provides data to FSIS establishing suitability and efficacy, FSIS would not object to the use of pullulan as a binder in various non-standardized meat products, provided that pullulan does not exceed 3.5 percent of the product formulation. Currently, there are no allowances for the use of pullulan as a binder in standardized meat products.

FSIS requested that FDA advise Hayashibara to seek regulatory guidance from FSIS, Labeling and Consumer Protection Staff, about the use of pullulan in meat products. Hayashibara should direct such an inquiry to Dr. Robert Post, Director, Labeling and Consumer Protection Staff, Office of Policy, Program Development and Evaluation, Food Safety and Inspection Service, 1400 Independence Ave., S.W., Suite 602, Annex, Washington, DC 20250-3700. The telephone number for his office is (202) 205-0279 and the telefax number is (202) 205-3625.

Sincerely,
/s/
Alan M. Rulis, Ph.D.
Director
Office of Food Additive Safety
center for Food Safety and Applied Nutrition

cc: Dr. Robert Post, Director
Labeling and Consumer Protection Staff
Office of Policy, Program Development and Evaluation
Food Safety and Inspection Service
1400 Independence Ave., S.W., Suite 602, Annex
Washington, DC 20250-3700

(1) Hayashibara considers that a twenty year history of safe use in Japan as a food ingredient and as a pharmaceutical bulking agent corroborates its view that pullulan would be safe under the conditions of its intended use.

(2) FDA independently estimated that daily intake of pullulan based on food categories and usage levels provided by Hayashibara would be 10 g/p/d at the mean and 20 g/p/d at the 90th percentile.

[Food Ingredients and Packaging](#) | [Summary of all GRAS Notices](#)

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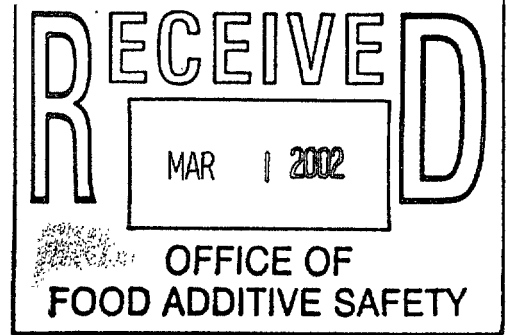
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Original Submission

000019



Pullulan GRAS Notification

Hayashibara International Inc.

8670 Wolff Court, Suite 200
Westminster, Colorado 80030
Tel: 303-650-4590
Fax: 303-650-9860

Provided by:

Lee B. Dexter and Assoc.
Technology Consultants
15704 Webberville Road
Austin, TX 78724
Tel: 512-276-7408
Fax: 512-276-7489

February 5, 2002

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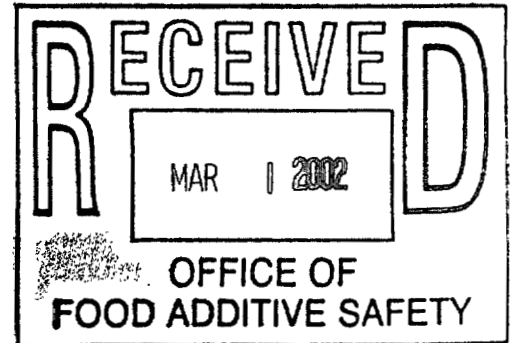
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Appendix A
HAYASHIBARA INTERNATIONAL INC.

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.

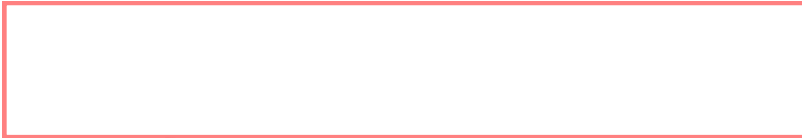
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**HAYASHIBARA
GROUP**

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.

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Claim

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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-toxicogenic 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 maltodextrans. 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

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Pullulan GRAS Notification**

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Section II

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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:

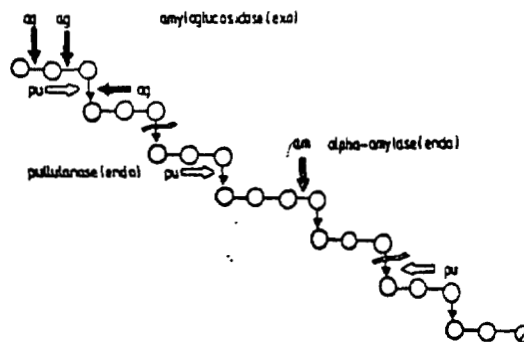
From: [Catley, *et al.*, 1986 Vol 2 Tab 5]

Fig. 1. The linear structure of pullulan, showing maltotriose units with the occasional replacement by maltotetraose: O, α -D-Glc; —, (1 \rightarrow 4) linkages; and |, (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 alpha-amyrase (am) acts randomly on the terminal (1 \rightarrow 4) linkage of maltotetraosyl residues.

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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.

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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)

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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

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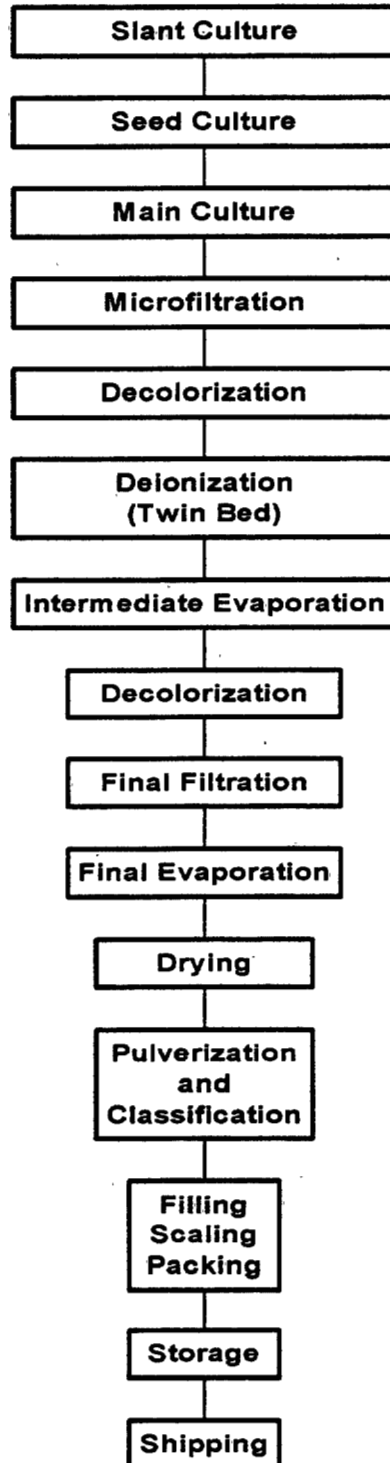
of the culture is confirmed, one colony is transferred to an agar slant. This colony is then used as the inoculum for the production of Pullulan.

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.

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Figure 1
Process Flow Diagram
For Hayashibara Pullulan



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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.

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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

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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.

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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

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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

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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

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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).

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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

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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	
<i>Salmonella</i>	Neg/25g	0	0	0	0	0	0	0	0	0	0	0	0	0	
<i>E.coli</i>	Neg/25g	0	0	0	0	0	0	0	0	0	0	0	0	0	
<i>S.aureus</i>	Neg/25g	0	0	0	0	0	0	0	0	0	0	0	0	0	

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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

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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

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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.

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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.

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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

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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

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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. Technical Effects, Intended Uses, Consumption Estimates, and Information on Self-Limiting LevelsSection 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.

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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).

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(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 Category ¹	Use Level (%)	Physical or Technical Functional Effect ²
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

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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 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

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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.

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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

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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

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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

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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

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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

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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

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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].

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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.

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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.

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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.

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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.

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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	

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(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)

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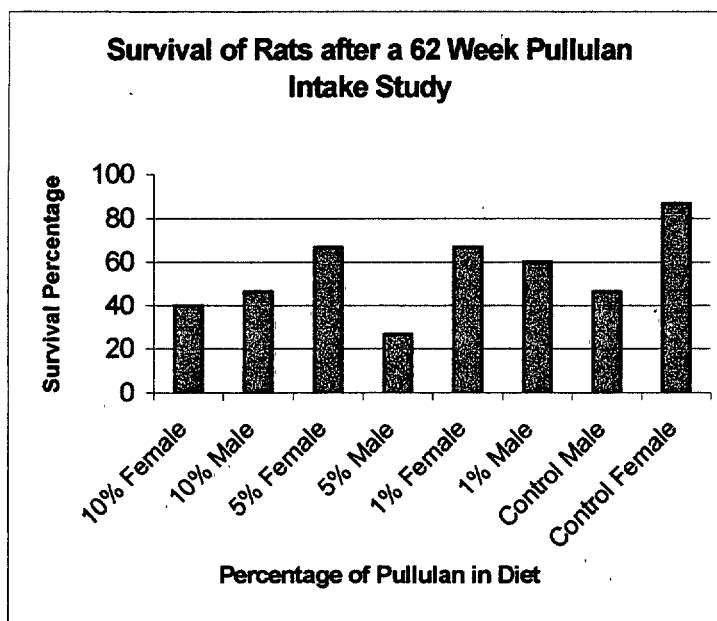
Other than the respiratory related findings (bronchitis, pneumonia, abscesses and adhesions) the following is a list of other conditions found in the various treatment groups. Unless otherwise stated (number of rats), each group represents one animal. Males: Heart: localized myocarditis, 1%, 5% and control: 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.

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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].

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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

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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].

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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

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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

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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.

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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

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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].

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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

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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

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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

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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.
Vice President & General Manager
Hayashibara International Inc.
8670 Wolff Court, Suite 200
Westminster, Colorado 80031
Tel 303-650-4590
Fax 303-650-9860

FROM: Donald G. Ahearn, Ph.D.
Professor of Microbiology
Georgia State University

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.

UNIVERSITY OF ILLINOIS
AT URBANA-CHAMPAIGN

Department of Animal Sciences
College of Agricultural, Consumer and Environmental Sciences
132 Animal Sciences Laboratory
1207 West Gregory Drive
Urbana, IL 61801

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]
000104

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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[Redacted Name Box]

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Medical College of Virginia

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August 20, 2001



MEMORANDUM

TO: Alan B. Richards, Ph.D.
Vice President & General Manager
Hayashibara International Inc.
8670 Wolff Court, Suite 200
Westminster, Colorado 80031
Tel 303-650-4590
Fax 303-650-9860

FROM: Donald G. Ahearn, Ph.D.
Professor of Microbiology
Georgia State University

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 *Exophialia* 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.

UNIVERSITY OF ILLINOIS
AT URBANA-CHAMPAIGN

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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,



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

GCF/hs

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UNIVERSITY OF ILLINOIS
AT URBANA-CHAMPAIGN

Department of Animal 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

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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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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

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Pagination**



Computer Technology Services, Inc.

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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

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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	"	"

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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	"	"

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Pullulan GRAS Notification

Hayashibara International Inc.

February 5, 2002

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

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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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.

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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.

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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

PULLULAN

Chemical and Technical Assessment (CTA)

prepared by Ivan Stankovic

1 Summary

Pullulan is a polysaccharide produced by a yeast like fungus *Aureobasidium pullulans*. Pullulan is an essentially linear glucan consisting mainly of 1,6-linked maltotriose and some interspersed maltotetraose units.

Pullulan has not previously been evaluated by JECFA but has been recommended for priority evaluation by CCFAC.

The commercially available pullulan (Pullulan PI-20) has a purity of more than 90 %. Its average molecular weight (at peak of a gel permeation chromatogram) is about 200 kD. The main impurities are mono-, di- and oligosaccharides, which are carried over from the raw material (hydrolysed starch) into the final product. The specifications for Pullulan include standard parameters for identification and for chemical and microbiological purity.

The film-forming properties of pullulan are the basis for its proposed use as a substitute for gelatin in the production of capsule shells (for dietary supplements), as an ingredient of coated tablets (dietary supplements), and as an ingredient of edible flavoured films (breath fresheners). It has been used as an additive and as a food ingredient in Japan since 1976.

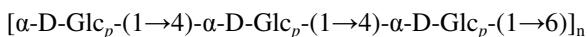
Specifications for Pullulan were prepared at 65th JECFA (2005) and will be published in FNP 52 Add 13.

2 Description**2.1 Chemistry and nature of the product**

The name "pullulan" was proposed by Bender, who was the first to describe the formation of this extra-cellular polysaccharide by *Aureobasidium pullulans* (syn. *Pullularia pullulans*) (Bender *et al.*, 1959). It is essentially a linear polymer of repeating maltotriose units linked by α -1,6 glycosidic bonds. Depending upon the culture conditions (duration, pH, phosphate concentration, etc.) under which this extra-cellular glucan is elaborated by *Aureobasidium pullulans*, the molecular weight varies from about 10 to 3 000 kDa (Sugimoto, 1978; Wiley *et al.*, 1993; Gibbs & Seviour, 1996; Madi *et al.*, 1997; Lazaridou *et al.*, 2002).

"Pullulan PI-20" is the brand name where "P" stands for "pullulan", "I" for "deionized" and the figure 20 designates the number-average molecular weight (M_n) of about 200 kDa (Okada *et al.*, 1990; Nakamura, 1984). The subject of the present CTA is pullulan (Pullulan PI-20) with a number-average molecular weight (M_n) of about 100-200 kDa and a weight-average molecular weight (M_w) of about 362-480 kDa (Okada *et al.*, 1990).

The INS No. of pullulan is 1204, the CAS No. is 9057-02-7, and its chemical formula is $(C_6H_{10}O_5)_n$. For Pullulan PI-20, n corresponds to about 1250 glucose units on the basis of M_n . The structural formula of pullulan corresponds to:



*Chemical and Technical Assessment 65th JECFA***2.2. Natural vs. synthetic origin**

Pullulan is a naturally occurring, fungal exopolysaccharide produced by *Aureobasidium pullulans*. The organism is ubiquitous. It is found in soil, lake water, on the surface of latex paint films, synthetic plastic materials, shared-used cosmetic and foods such as cereals, fruits, cheese and tomato (Vadkertiova 1964, Zabel *et al.*, 1980, Webb *et al.*, 1999, Mislivec *et al.*, 1993). Because it forms a black pigment (melanin), this organism is also known as "black yeast" (Cooke, 1961, Durrell, 1967, Domsch *et al.*, 1993; Gibbs & Seviour, 1996). Pullulan is produced on an industrial scale by fermentation of liquefied starch under controlled conditions using a specific, not genetically modified, non-pathogenic and non-toxicogenic strain of *Aureobasidium pullulans*.

3 Method of manufacture**3.1 Principle**

Pullulan is produced commercially by mesophilic (22-30°C) fermentation of hydrolysed starch with a selected non-toxicogenic strain of *Aureobasidium pullulans* (Yuen, 1974; Leathers, 2003; Shingel, 2004) and further purification of the product. Pullulan is formed extracellularly when the cells are in the late log phase and stationary phase of growth (Catley, 1971) and its formation is dependent on a various factors including pH, temperature, substrate and strain (Catley, 1971, Yuen, 1974, Madi *et al.*, 1997, Gibbs and Seviour, 1996, Lazaridou *et al.* 2002, Sugimoto, 1978, Ueda *et al.* 1963, 1966). The yield and molecular weight of pullulan can be adjusted by manipulation of the substrate and fermentation conditions.

3.2 Detailed description

The manufacturing process is conducted under conditions of good manufacturing practices and uses raw materials and processing aids that comply with food grade specifications. Pullulan is produced by mesophilic fermentation of starch syrup by the selected non-toxicogenic strain of *Aureobasidium pullulans*. The strain has been selected by traditional techniques, i.e. the strain is not the product of genetic modification using recombinant technologies. The production strain has a high yield of pullulan, low production of melanin and does not produce aureobasidin A.

After completion of the fermentation, the fungal cells are removed by microfiltration. The cell-free filtrate is heat-sterilized and treated with activated carbon to remove pigments and other impurities by adsorption. The decolourized filtrate is cooled and deionized using cation and anion exchange resins. The deionized solution is concentrated to a solids content of about 12 %, treated a second time with activated carbon, and filtered using diatomaceous earth as a filter aid. The filtrate is concentrated by evaporation to a solids content of about 30 % and dried in a drum dryer. The dried pullulan is pulverized to a specified particle size and packed in sterilized polyethylene bags.

4 Chemical characterization**4.1 Composition of pullulan**

Pullulan is an essentially linear polysaccharide (glucan) consisting predominantly of repeating maltotriose units. The maltotriose units, which consist of three 1,4-linked glucose molecules, are linked by α -1,6-glycosidic bonds. This repeating sequence forms a stair-step-type structure (Figure 1).

Chemical and Technical Assessment 65th JECFA

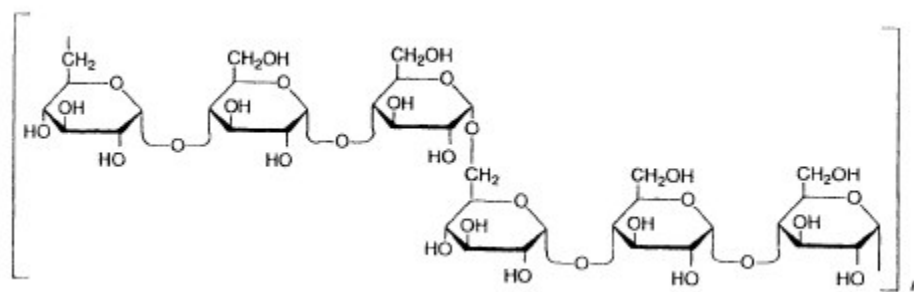


Figure 1. Structural formula of pullulan

Maltotetraose units consisting of four 1,4-linked glucose molecules also occur, probably randomly, but are rarer (about 6 %) (Wallenfels *et al.*, 1965, Catley, 1971; Carolan *et al.*, 1983). There is also evidence for a rare occurrence of branching points where poly-maltotriosyl side-chains are attached to the main chain by a 1,3-glycosidic bond (Catley *et al.*, 1986; Sowa *et al.*, 1963).

4.2 Physicochemical properties

Pullulan PI-20 is a white to off-white, tasteless and odourless powder. It is not hygroscopic. According to the specifications provided by the sponsors it contains less than 6 % water. Pullulan dissolves readily in cold or hot water, but is insoluble in organic solvents, except dimethylformamide and dimethylsulfoxide (Sugimoto, 1978; Tsujisaka & Mitsuhashi, 1993). Aqueous solutions of pullulan are viscous but do not form gels. The viscosity (10% w/w, 30°C) of ten batches of pullulan PI-20 was 132-179 mm²/s. The viscosity of pullulan solutions resembles that of gum acacia (gum arabic) solutions, i.e. the viscosity of pullulan is rather low in comparison with that of other soluble polysaccharides, such as guar gum (Sugimoto, 1978; Tsujisaka & Mitsuhashi, 1993). Differences in the pH or salt content do not substantially affect the viscosity of pullulan solutions (Sugimoto, 1978). The viscosity of an aqueous solution of Pullulan PI-20 decreases upon incubation with pullulanase (EC 3.2.1.41). An aqueous solution of Pullulan PI-20 (10% w/w) has a pH of 5.0-7.0.

Molecular weight gel permeation chromatograms of three batches of Pullulan PI-20 showed a molecular weight at the peak of the chromatogram of 173000-186000 Da with a number-average molecular weight (M_n) of 96900-101000 Da and a weight-average molecular weight (M_w) of 433000-479000 Da.

Pullulan is structurally closely related to starch amylopectin and maltodextrin. All three carbohydrates consist of glucose units linked by α -1,4 and α -1,6-glycosidic bonds. Maltodextrin contains approximately 20% α -1,6-glycosidic bonds and pullulan approximately 30%. In comparison, corn starch contains 95% α -1,4-glycosidic bonds and 5% α -1,6-glycosidic bonds. Differences between pullulan and these glucans, besides the relative proportions of α -1,4 and α -1,6 bonds, are the tertiary structure of the molecule and the extent and mechanism of degradation of the materials in the human gut. Pullulan can be classified in the group of soluble fibres.

4.3 Possible impurities (including degradation products)

Pullulan PI-20 has a purity of more than 90% on a dry substance basis. Since the purity of this polymeric substance cannot be determined directly, it is calculated as the difference between 100% and the sum of the percentages of analytically determined known impurities, i.e. mono-, di- and oligosaccharides (determined with anthrone-sulfuric acid reagent (Morris, 1948)) and water. The purity of 10 batches of Pullulan PI-20 was in the range of 91.2- 95.0 %.

Chemical and Technical Assessment 65th JECFA

The analysis of 10 lots of Pullulan PI-20 reveals a combined content of mono-, di- and oligosaccharides of between 5.0 and 8.7%. Mono- and disaccharides constitute about 30-40 % of these non-pullulan carbohydrates. About 37-42% of the molecules have a degree of polymerization (DP) between 3 and 10. These carbohydrates are derived from the food-grade corn syrup, which is used as the raw material for the production of pullulan.

The analysis of 10 lots of Pullulan PI-20 reveals ash contents of 0.00-0.16 %. The sources of minerals are the food-grade corn syrup raw material and the inorganic salts, which are added to the culture medium (ammonium sulfate, calcium hydroxide, sodium chloride, diammonium phosphate, dipotassium phosphate, sodium glutamate, magnesium sulfate).

The analysis of 10 lots of Pullulan PI-20 using the semi-micro-Kjeldahl method reveals a nitrogen content of 0.002-0.004% (limit of detection: 0.001%). Applying the standard conversion factor of 6.25, this corresponds to a protein content of about 0.01-0.03 %.

Specific analyses for lead revealed values of less than 1 mg/kg, i.e. lower than general JECFA limit of 2 mg/kg. Additional analyses of two different batches of Pullulan PI-20 for Cd, Pb, Hg and As confirmed the high purity of the product.

Other impurities in pullulan could stem either from the starting material (food-grade corn syrup), the fermentative action and metabolism of *Aureobasidium pullulans*, or the cell wall of this microorganism. The purification steps that are included in the manufacturing process of Pullulan PI-20 ensure that such by-products are eliminated. Thus particulate materials from the cell walls are removed by micro filtration and treatment with activated carbon; ionic compounds (e.g. fermentatively produced organic acids) are removed during the deionization step; organic compounds (e.g. melanin, protein) are absorbed during the treatment with activated carbon; and volatile products (e.g. fermentatively formed ethanol) disappear during the final evaporation and drying.

Two batches of Pullulan PI-20 were subjected to detailed microbiological analysis. With the exception of the test for so called 'flat sour spores', none of the applied tests gave a result above the threshold of detection. Flat sour spores were found in one of the two tested batches only at 2 CFU/g. Flat sour spores represent mainly *Bacillus stearothermophilus* and *Bacillus coagulans*. It is likely that these heat-resistant, spore-forming microorganisms originate from the raw material, i.e. corn syrup. The canning industry, for example, accepts sugar with flat sours of up to 7.5 CFU/g.

There was no evidence for the presence of *Aureobasidium pullulans* in ten examined batches. This microorganism would be detected in the tests for yeast and moulds. Analyses for total mesophilic bacteria demonstrate the high microbiological purity of the product.

Some strains of *Aureobasidium pullulans* produce aureobasidin A, which is toxic to fungi and yeast at low concentrations (0.1-0.5 µg/ml). The strain used for the production of pullulan has been checked for aureobasidin production. Using *Saccharomyces cerevisiae* as a sensitive tester strain, it was determined that neither pullulan (samples from six commercial batches) nor the unpurified filtrate of the pullulan culture medium contained aureobasidin-like activity (limit of detection 2 ppm) (Hasimoto & Fukuda, 2002). There are no observations that would indicate that *Aureobasidium pullulans* produces mycotoxins other than aureobasidin. Two batches of Pullulan PI-20 were analysed for the presence of aflatoxins (B₁, B₂, G₁, G₂), zearalenone, sterigmatocystin and ochratoxin. Using standard analytical methods, all batches tested negatively for these mycotoxins.

Tests of two batches of Pullulan PI-20 with a number of tester strains for antibacterial activity gave negative results.

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4.4 Analytical methods

The analytical methods for the proposed specifications of pullulan are based on general tests for identity and purity published in Food and Nutrition Paper 5, Rev 2 (FAO, 1991) (solubility, pH, sulfated ash, loss on drying, lead, nitrogen determination, microbiological criteria and spectrophotometry), as well as the determination of mono-, di- and oligosaccharides with Dreywood's anthrone reagent (Morris, 1948) and determination of kinematic viscosity using a Ubbelohde-type (falling-ball) viscometer.

4.5 Rationale for proposed specifications

The specifications proposed for Pullulan are based on the manufacturing process and raw materials and define the composition of the material of commerce. The parameters tested include the identified components of pullulan. Specifications of polymeric carbohydrates take into consideration the heterogeneous composition of these products with respect to chain length, degree of branching, etc. Pullulan is a glucan, i.e. a homopolysaccharide consisting solely of glucose molecules, and the definition of the purity of pullulan as 'not less than 90% of glucan' is, therefore, consistent with current practice. Batches containing less than 90% of glucan on an anhydrous basis would not meet the proposed specifications. Levels of possible impurities are also included in the specifications to ensure that these levels remain at a minimum and that the article of commerce is identical to that evaluated in the toxicological tests. The lead limit is included in the specifications for safety purposes and is lower than the general limit adopted by JECFA. In addition, analytical data for 10 different manufacturing batches of Pullulan PI-20 indicate that the method of manufacture produces a consistent product and suggests that the finished product produced by the described manufacturing process complies with the proposed specifications.

5 Functional uses

Pullulan is used as a glazing agent, as a film forming agent, as a thickener or as a carrier in the production of capsules for dietary supplements (substitute for gelatin), coatings for coated tablets (dietary supplements), for production of edible flavoured films (breath fresheners), jams and jellies, confectionery and some meat and fruit products. It is also used as a texturizer in chewing gum and as a foaming agent in milk based desserts.

5.1 Technological function

Pullulan forms transparent, water-soluble, fat-resistant, antistatic films of low oxygen permeability (Tsujisaka & Mitsuhashi, 1993; Sugimoto, 1978; Yuen, 1974). Films are usually prepared by rapid evaporation of a 5-10% aqueous pullulan solution applied to a smooth surface and dried. Very thin films (down to 0.01mm) can be made (Yuan, 1974). By admixture of other components, the relevant properties of these films can be modified (Yuen, 1974; Shih, 1996; Biliaderis *et al.*, 1999; Diab *et al.*, 2001). By compression moulding or extrusion at elevated temperature, pullulan films can be formed to shaped bodies (Hijiya & Shiosaka, 1974). Because of these properties, Pullulan PI-20 can be used as a substitute for gelatin in the production of capsule shells for dietary supplements and medicinal products.

Pullulan-based edible films can also serve as a matrix to hold flavours. Pullulan films with, for example, menthol dissolve quickly on consumption, releasing the bound flavour and thus acting as an instant breath freshener. Because pullulan-based films have a low permeability to oxygen and humidity, pullulan may also be used for the coating of foods in tablet form (dietary supplements). In this application, it protects susceptible ingredients (nutrients, colours, flavours) from deterioration and thus preserves the nutritional and organoleptic quality of the products.

5.2 Food Categories and use levels

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Pullulan-based hard or capsule shells may contain 15-90% pullulan. Pullulan-based, flavoured, edible films consumed as breath fresheners may contain up to 90% pullulan. The coating of tablets with pullulan results in products with a content of up to 2% pullulan. Use levels of pullulan in jams and jellies, chewing gum, confectionery and some meat, milk and fruit products are in the range of 0.2 to 5%.

6 Reactions and Fate in Foods

6.1 Stability

The chemical structure and thus the reactivity of pullulan resembles that of maltodextrin and starch, both of which are common constituents of food. Having a large molecular weight, Pullulan PI-20 is essentially non-reducing. It is stable in aqueous solution over a wide pH range (3-8) (Wallenfels *et al.*, 1965). Only prolonged heating at pH < 3 leads to a decrease of viscosity which is indicative of hydrolytic depolymerization (Nakamura, 1984).

On dry heating, pullulan decomposes and carbonizes at 250-280°C (Tsujisaka & Mitsuhashi, 1993).

6.2 Chemical interactions with nutrients

Because it does not contain any chemically reactive group, pullulan is not expected to interact chemically with other nutrients in foods. As it is not degraded by the digestive enzymes of the human alimentary tract to a significant extent, pullulan remains intact in the small intestine. Having a low viscosity and a chemical structure lacking anionic or cationic groups at recommended levels of intake, pullulan is not expected to impair the small-intestinal absorption of essential nutrients such as vitamins and minerals (Gordon *et al.*, 1995, Gorman & Bowman, 1993, Rossander *et al.*, 1992, Kelsay, 1990).

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PULLULAN

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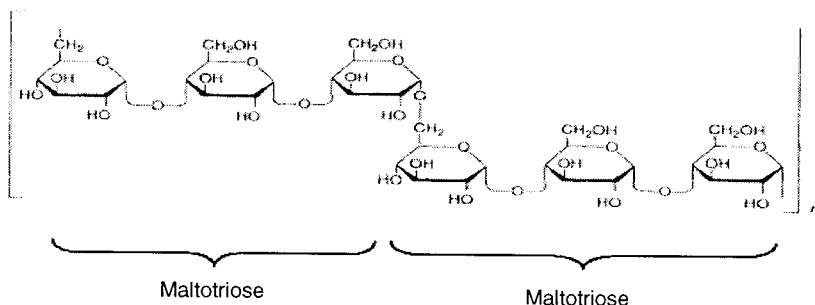
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1. EXPLANATION

Pullulan is a naturally occurring, fungal polysaccharide produced by fermentation of liquefied corn starch by *Aureobasidium pullulans*, a ubiquitous yeast-like fungus. It has a linear structure consisting predominantly of repeating maltotriose units, which are made up of three α -1,4-linked glucose molecules (Wallenfels et al., 1965; Catley, 1971; Carolan et al., 1983), linked by α -1,6-glycosidic bonds. The maltotriose units are interspersed with about 6% maltotetraose units consisting of four α -1,4-linked glucose molecules; rarely, branch points occur, at which poly-maltotriosyl side-chains are attached to the main chain by a 1,3-glycosidic bond (Figure 1; Sowa et al., 1963; Catley et al., 1986).

Pullulan is used as a glazing agent, as a film-forming agent, as a thickener or as a carrier in the production of capsules for dietary supplements as a substitute for gelatin, coatings for coated tablets containing dietary supplements, for production of edible flavoured films used as breath fresheners, and in the production of jams and jellies, confectionery and some meat and fruit products. It is also used as a texturizer in chewing-gum and as a foaming agent in milk-based desserts (Sugimoto, 1978; Wiley et al., 1993; Gibbs & Seviour, 1996; Madi et al., 1997; Lazaridou et al., 2002).

Figure 1. Structure of pullulan



The Codex Committee on Food Additives and Contaminants at its Thirty-sixth Session (Alinorm 04/27/12) asked the Committee to review pullulan. This substance has not been evaluated previously by the Committee.

Pullulan is produced by fermentation from a food-grade hydrolysed starch with a non-toxin-producing strain of *Aureobasidium pullulans*. After fermentation, the fungal biomass is removed by microfiltration, the filtrate is heat-sterilized, and pigments and other impurities are removed by adsorption and ion-exchange chromatography. The product contains not less than 90% glucan on a dried basis. The main impurities are mono-, di- and oligosaccharides from the starting material. The average relative molecular mass of pullulan varies considerably, depending on culture conditions. A commercially available product has an average relative molecular mass of 200 000 Da.

Pullulan is stable in aqueous solution over a wide pH range (pH 3–8). Pullulan decomposes upon dry heating and carbonizes at 250–280 °C. It dissolves readily in water but is insoluble in organic solvents. Aqueous solutions of pullulan are viscous but do not form gels. Upon drying, pullulan forms transparent, water-soluble, fat-resistant, odourless, anti-static, flavourless films.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Absorption, distribution, biotransformation and excretion

Early experiments on the digestibility of pullulan by human salivary amylase and hog pancreatic amylase in vitro demonstrated that pullulan is either hydrolysed either slowly or not at all by these enzymes (Ueda et al., 1963; Wallenfels et al., 1965).

In another study on the digestibility of pullulan, two samples of relative molecular mass of 50 000 and 200 000 Da were treated sequentially with human salivary amylase, porcine pancreatic amylase, artificial gastric juice and an enzyme preparation from the small intestinal mucosa of rats. The 50 000-Da pullulan was

hydrolysed by the intestinal enzymes to produce 2.7% glucose but was not affected by the other treatments. The 200 000-Da pullulan was sequentially converted to substances of lower relative molecular mass (average, 70 000 Da) after treatment with the intestinal enzyme preparation; no glucose was released after salivary or pancreatic amylase treatment, but a small increase in reducing sugar content was observed (0.6% and 0.7%, respectively). A 6.6% increase in glucose concentration was observed after digestion with the intestinal enzyme preparation. The authors suggested that glucose is formed as a result of hydrolysis of the α -1,4-glycosidic bond from the non-reducing end of the molecule, but that the hydrolysis stops at the α -1,6-glycosidic bond. The amount of glucose released from pullulan standards (relative molecular mass, 990–380 000 Da) digested in vitro in the small intestinal enzyme preparation ranged from 1.5% (relative molecular mass, > 100 000 Da) to 36.3% for the smallest sample (990 Da) (Okada et al., 1990).

In a study to determine the digestibility of pullulan film (relative molecular mass unspecified), a 1% pullulan solution was treated with an enzyme mix containing α -amylase, amyloglucosidase, peptidase, protease, invertase and lipase for up to 2 h. About 9% pullulan, < 5% levan powder and cellulose film and > 90% starch powder and starch film were hydrolysed (Kunkel & Seo, 1994).

In another study of the digestion of pullulan (relative molecular mass, 10 000 Da), 37% raw pullulan and 42% cooked substance were hydrolysed in vitro by α -amylase and amyloglucosidase within 30 min. Control samples of raw and cooked maltodextrin were completely hydrolysed during this time. Hydrolysis of the remaining pullulan proceeded more slowly, reaching 95% completion after 5 h (Wolf et al., 2003).

In a further study on the digestion of orally administered pullulan, five fasted male Wistar rats were given 2 ml of a 10% pullulan solution in 0.9% saline by gavage. The pullulan used in this study had a relative molecular mass of 49 000 Da and consisted of 302 glucose molecules; 93% of the glucose units were in the form of maltotriose and 7% in the form of maltotetraose. The animals were killed 1 h after gavage, and the contents of their stomachs and small intestines were collected, homogenized and analysed for glucose to determine the extent of pullulan hydrolysis. The glucose concentrations in homogenates of pullulan-treated animals suggested that about 3% of the pullulan had been hydrolysed; however, it was not known if the hydrolysis products of pullulan were absorbed by the small intestine. The finding that about 3% pullulan was hydrolysed was close to the estimate that 2.5% would be hydrolysed, on the basis of 7% maltotetraose units containing one α -1,4-glycosidic bond susceptible to amylase. Nevertheless, low glucoamylase activity was present in the intestinal tract, which can slowly hydrolyse α -1,4- and α -1,6-glycosidic bonds from the non-reducing end (Oku et al., 1979).

In a study to determine the rate and extent of disappearance of starch from the small intestine, groups of seven Sprague-Dawley rats (two groups per treatment) were fed pullulan, cornstarch, maltodextrin, modified maltodextrin or amylo maize and then killed; their small intestines were then removed and clamped to give 15 equal-sized portions. The contents of the small intestines were expressed and precipitated in ethanol. Starch disappearance, expressed as total starch, was measured in each of the 15 intestinal segments. Pullulan (average relative molecular mass, 10 000 Da) disappeared gradually, reaching a maximum disappearance of 81.4 g/100 g pullulan at segment 13. The authors noted that, with this method, all

pullulan and its products that are soluble in ethanol would be considered to be digested and that the estimate of pullulan digestion might be exaggerated (Bauer et al., 2003).

In a study on the effects of caecal microflora on pullulan, the concentration of short-chain fatty acids was significantly greater in rats fed diets containing 10% pullulan for 4 weeks than in control rats fed diets containing 5% cornstarch (Sugawa-Katayama et al., 1994).

Another study showed that pullulan (average relative molecular mass, 50 000 Da) is fully digested in human faecal cultures within 4–8 h, yielding a maximum of 52.7 g short-chain fatty acids/100 g pullulan (mainly acetic, propionic and butyric acids). The energy value for pullulan was estimated to be 2.05 kcal/g, assuming absorption of 100% short-chain fatty acids; however, with increasing pullulan intake, it is unlikely that all short-chain fatty acids produced will be absorbed (Okada et al., 1990).

In a study of the digestion of pullulan by intestinal bacteria, the compound was not detected in the faeces of six volunteers who had consumed 10 g pullulan (relative molecular mass, 50 000 Da) daily for 14 days. The short-chain fatty acid concentration in the faeces increased from 6 mg/g to 8.8 mg/g faeces. The authors concluded that pullulan is completely fermented to short-chain fatty acids by intestinal bacteria (Yoneyama et al., 1990).

The effect of pullulan on the postprandial glycaemic response of healthy non-diabetic adults was compared with that of maltodextrin, a rapidly absorbed starch that normally elicits a high glycaemic response. In a randomized, double-blind, two-period, two-treatment, cross-over meal tolerance test, 28 volunteers (19 men and 9 women) were asked to eat a high-carbohydrate diet for 3 days and to avoid exercise for 24 h before testing. After an overnight fast, the volunteers were given a sterilized flavoured drink containing either 50 g pullulan (relative molecular mass, 100 000 Da) or 50 g maltodextrin. Blood glucose was measured in finger-prick blood before treatment, every 15 min for the first hour and then every 30 min up to 180 min. Carbohydrate absorption was measured by analysing breath hydrogen every hour for 8 h. The persons were asked to report any symptoms of nausea, abdominal cramping, distension or flatulence for 48 h after treatment. The cross-over treatment was carried out 5–13 days after the first treatment. The postprandial blood glucose concentration (1.97 mmol/l) was significantly lower in persons who ate pullulan than in those who ate maltodextrin (4.24 mmol/l), and the time to peak glucose concentration was delayed in the pullulan-treated group. Carbohydrate malabsorption was greater in persons taking pullulan. Flatulence was the main side-effect and was commonest during the first 24 h after treatment. The authors concluded that pullulan is slowly digested in the human gut (Wolf et al., 2003).

In a study to determine the glycaemic index of pullulan (average relative molecular mass, 200 000 Da) after a 12-h fast, five volunteers (one with non-insulin-dependent diabetes) were given a bolus dose of 25 g pullulan. A 25-g dose of maltose was taken 6 days later as a positive control. Blood glucose concentrations were determined before dosing and 15, 30, 45, 60, 90 and 180 min after dosing. The glycaemic response to pullulan relative to that to maltose was 12.8% when all persons were considered, but the one diabetic person, who had an exaggerated response to maltose, influenced this response. When this person was excluded, the relative glycaemic index for pullulan was 18.6%. Statistical comparisons at each sampling

time showed that the blood glucose values for the maltose control at 30 and 45 min were significantly greater than those for pullulan ($p < 0.01$). The mean area under the curve of concentration:time value for the control (103 ± 37) was significantly greater ($p < 0.02$) than that for pullulan (19 ± 13) (Richards & Higashiyama, 2004).

In 1990, a United States patent indicated that pullulan reduces peak blood glucose concentrations when taken with food products containing starch or sucrose at a ratio of pullulan:starch or pullulan:sucrose of 1:400 to 1:20; however, the effect was not consistent and varied with dose, the relative molecular mass of the pullulan and the age and health of the persons. In the absence of a mechanistic explanation for this finding, the conclusions are questionable (Hiji, 1990).

Another study showed that pullulan had no effect on the blood glucose concentrations (measured every 30 min from 0 to 180 min) of a 39-year-old volunteer given a solution of 50 g glucose containing 0, 5 or 10 g pullulan in 200 ml water (Oku et al., 1983).

Overall, the studies indicate that pullulan is hydrolysed only very slowly by gastrointestinal enzymes but is fermented by intestinal microorganisms to short-chain fatty acids. In humans, the glycaemic response for pullulan relative to maltose was 18.6%.

2.1.2 Effects on enzymes and other biochemical parameters

The possibility that large amounts of indigestible carbohydrate in the diet can decrease vitamin and mineral absorption has been addressed in several review articles. It has generally been accepted that the presence of dietary fibre at recommended levels in the diet does not adversely affect vitamin and mineral status (Kelsay, 1990; Rossander et al., 1992; Gorman & Bowman, 1993). Even when dietary fibre was consumed in large amounts (50 g per day), no adverse effects on mineral absorption or nutrition were observed (Gordon et al., 1995).

One study on the inhibitory effect of pullulan on intestinal calcium absorption was conducted in groups of six male Wistar rats fed diets containing 20% pullulan (relative molecular mass unspecified), another unavailable carbohydrate (cellulose or glucomannin) or a control diet containing cornstarch for 8 weeks. Rats were fasted for 16 h before sacrifice, and then a homogenate of duodenal mucosa was prepared. The calcium-binding activity of the duodenal supernatant was measured, as were serum calcium concentrations. Calcium-binding activity was significantly reduced in animals fed diets containing 20% pullulan; however, there was no significant difference in serum calcium. Alkaline phosphatase and sucrase activity in the duodenum were also reduced (by approximately one-half and one-third, respectively) from that of the control group. Nevertheless, the parameters were similar between groups receiving unavailable carbohydrate-containing diets. The authors suggested that the inhibitory effect of unavailable carbohydrate on intestinal calcium absorption is due partly to loss of calcium-binding proteins by gastrointestinal transit of large amounts of undigested substances (Oku et al., 1982).

2.2 Toxicological studies

2.2.1 Acute toxicity

The acute toxicity of pullulan (quality and relative molecular mass unspecified) was examined in one study in male mice (number per group unspecified). No

information was given on GLP or other guidelines used. The LD₅₀ was > 14 g/kg bw (Department of Public Hygiene, 1974a).

2.2.2 Short-term studies of toxicity

Rats

In a study (no information on GLP supplied) on the effects of pullulan on the digestive tract, groups of eight male Wistar rats were fed diets containing 0, 5%, 10%, 20% or 40% pullulan, equivalent to 0, 2500, 5000, 10 000 and 20 000 mg/kg bw per day, for 4 or 9 weeks. Another group of rats were fed diets containing 20% and 40% cellulose as a comparison. Body-weight gains were reduced by day 10 in the rats given 20% or 40% pullulan and by day 20 in that fed 40% cellulose. The weight differences increased throughout the remainder of the study. The weight gain of animals at 5% or 10% and that of rats given 20% cellulose were also lower than those of controls after 7 weeks, although the difference was not statistically significant. Diarrhoea was observed at 40% pullulan, but the number of rats affected and the frequency were not reported. Unlike maltitol-induced diarrhoea, it did not resolve after a period of adaptation and occurred only occasionally throughout the study. The relative weight of the caecum was increased in a dose-related manner in rats given pullulan. In general, the relative weights of the stomach, small intestine and large intestine were increased in treated animals (Oku et al., 1979).

In a study on the toxicity of an unspecified polysaccharide produced by *A. pullulans*, groups of 10 male and female Wistar rats received an oral dose of 2.5 or 25 mg/kg bw per day of the substance daily for 7 weeks. No adverse effects were reported (Fujii & Shinohara, 1986).

In a study on changes in the colon mucosa of rats fed pullulan, groups of eight 6-week-old male Sprague-Dawley rats were fed diets containing 1% or 10% pullulan (relative molecular mass not specified), equivalent to 500 and 5000 mg/kg bw per day, for 4 weeks. A control group of rats was fed a diet containing 5% cellulose (equivalent to 2500 mg/kg bw per day). Changes in the colon mucosa were analysed by scanning electron micrography and by comparing colon cell sizes (protein:DNA ratios). Scanning electron micrographs of the colons suggested that the haustra coli (pouches formed in the colon by muscle contractions of the colon walls) were broader in the pullulan-fed rats than in the control group. Faecal weight was significantly decreased in a dose-related manner in rats given pullulan. The wet weight of the colon mucosa was significantly increased in rats given 10% pullulan. The mucosal protein content (reported in mg/cm of colon) was decreased in rats at either concentration of pullulan but more markedly in those given 1%, and the DNA content was significantly increased in rats given 10% pullulan. The authors concluded that pullulan decreased the size of colon mucosa cells (Sugawa-Katayama et al., 1993).

In a study conducted according to GLP, groups of 10 SPF Wistar rats of each sex were fed diets containing pullulan (relative molecular mass, 200 000 Da) at a concentration of 0, 2.5%, 5% or 10%, equal to 0, 1960, 4100 and 7900 mg/kg bw per day. The control diet and those containing the two lower doses of pullulan were supplemented with potato starch (10%, 7.5% and 5%, respectively) to achieve 10% in the diet. The animals were examined daily for clinical signs of toxicity, and body weights and food consumption were recorded at regular intervals. Grip strength

and locomotor activity were measured in week 13. At the end of the 13-week treatment, urine and blood were collected from fasting animals for urinalysis, haematology and clinical chemistry. Animals were killed and organs and tissues examined macroscopically and weighed. Histological examinations were performed on the liver, kidney, caecum, duodenum, colon, ileum, jejunum, rectum, lungs, spleen and mesenteric and mandibular lymph nodes. No deaths occurred, and no treatment-related clinical signs were observed. Food consumption, food use and body weight were similar in all groups. There were some minor changes in grip strength in males, but this was not dose-related. Significantly reduced motor activity ($p < 0.05$) was observed in females at the medium dose (after 45 and 60 min) and in the group at the highest dose (after 60 min). This was reflected in total motor activity and appeared to be related to treatment; however, the changes seemed to reflect physiological phenomena due to unused carbohydrate in the diets, rather than to any toxic effects. Haematological analyses revealed no treatment-related effects. Males and females at the lowest dose showed slightly lower prothrombin activity. Males at the two lower doses showed increased activated partial thromboplastin time, and females at these doses had lower relative reticulocyte counts.

Clinical chemistry parameters showed a number of significant differences, males at the lowest dose having significantly decreased uric acid and sodium, males at the two lower doses significantly decreased cholesterol, phospholipids and potassium, males at the two higher doses significantly decreased calcium, males at the highest dose significantly increased plasma glucose and all treated males having significantly reduced globulin. Females at the two higher doses had significantly increased sodium, and females at the highest dose had decreased triglycerides. The authors reported that these values were within or marginally outside the reference range and therefore not biologically significant. Furthermore, the observed differences were not dose-dependent, nor were they observed in both sexes. There were no changes in urinary parameters in male rats at any dose; however, urine volume was significantly increased in females at the two higher doses (308% and 285% of control, respectively). As there were no concurrent changes in relative densities or in pH values, the urine volume changes were considered by the authors to be artificial rather than treatment-related effects. Dose-dependent increases in absolute and relative caecal weights were found in males and females, which was statistically significant in males at the two higher doses for empty caecum weight and for males at the intermediate dose for full caecum weight. In females, the increase was statistically significant at the highest dose for empty caecal weight, and at the two higher doses for the relative weight of the empty caecum. One male at the lowest, two at the intermediate and one at the highest dose had distended caecums. Other macroscopic findings consisted of renal pelvic dilatation (one male each at the two lower doses, one female at the intermediate dose and two females at the highest dose), diaphragmatic herniation of the liver (one male at the intermediate dose), uterus dilatation (one female each at the two higher doses), and a dark-red, discoloured focus in the thymus of one male at the lowest dose. No test-related microscopic changes were observed on histopathological examination. As caecal hypertrophy is considered to be a physiological response to poorly digested carbohydrates, a dietary level of 10% pullulan (equal to 7900 mg/kg bw per day) was tolerated by male and female rats without toxicological effects (Sommer et al., 2003).

2.2.3 Long-term studies of toxicity and carcinogenicity

In a test for toxicity not conducted under GLP, groups of 15 4-week-old Sprague-Dawley (SD-JCL) rats of each sex were fed diets containing pullulan (relative molecular mass not specified) at a concentration of 0, 1%, 5% or 10%, equivalent to 0, 500, 2500 and 5000 mg/kg bw per day, for 62 weeks. The study was intended to be conducted over 24 months but was terminated at 14 months (62 weeks) owing to high mortality in all groups as a result of infection. Animals were observed daily; body weights and food consumption were recorded weekly. At the end of the study, the animals were killed and blood and urine were taken for analysis. The blood was tested to determine the red blood cell count, haemoglobin concentration, haematocyte count, white blood cell count and percentage, serum aspartate and alanine aminotransferase and alkaline phosphatase activity, serum total cholesterol, serum cholinesterase activity, serum protein, albumin:globulin ratio and blood sugar. Urine was analysed for protein, sugar, ketones, pH and blood. Internal organs were weighed and examined macro- and microscopically.

The high mortality observed in all groups was attributed to pneumonia. Survival to the end of the study appeared to be dose-related in females (87%, 67%, 67% and 40% at the four doses of pullulan, respectively) but not in males (47%, 60%, 27% and 47%, respectively). No significant differences were observed between groups in terms of food intake or body-weight gain. The terminal body weights of males at 1% and 10% were significantly lower than those of the controls; however, this effect was not dose-related (89%, 96% and 91% of control at 1%, 5% and 10% pullulan, respectively). Some significant differences were observed in absolute organ weights. The absolute weight of the brain was decreased in females at the two lower doses and in males at the lowest dose. In females, the absolute weights of the heart, liver, spleen and caecum were increased at some doses; in males, the absolute weights of the heart, liver, kidneys, stomach and submandibular gland were decreased at some doses. No significant differences were reported in relative organ weights. The 46% increase in absolute caecal weight in females at the highest dose was attributed to a physiological response to undigested pullulan. No data were provided on male caecal weights. Post-mortem macroscopic examination of tissues revealed pneumonia and pulmonary abscesses in animals in all groups. Histopathological observations confirmed bronchitis in animals in all groups but did not indicate a dose-related effect. A few statistically significant differences were observed in haematological and clinical chemistry parameters, which were not dose-related. Urine analysis showed no significant differences in any group.

The changes seen between the control and test groups were not consistent or treatment-related; therefore, the NOEL was the highest dose tested, 10% pullulan in the diet (equal to 5000 mg/kg bw per day) (Kotani et al., 1976; Kimoto et al., 1997). The value of this study is limited because of the high mortality in all groups.

2.2.4 Genotoxicity

The results of studies on the genotoxicity of pullulan *in vitro* and *in vivo* are shown in Table 1.

Table 1. Results of assays for genotoxicity with pullulan

Test system	Test object	Concentration	Results	Reference
<i>In vitro</i>				
Reverse mutation ^a	<i>S. typhimurium</i> TA1535, TA100, TA1537, TA98	10–10 000 µg/plate	Negative ^a	Hatano Research Institute (1978); Kimoto et al. (1997)
DNA damage	<i>Bacillus subtilis</i>	20 mg/plate	Weakly positive ^b	Kuroda et al. (1989)
Chromosomal aberrations	Chinese hamster lung fibroblasts	12 mg/ml	Negative (after 48 h)	Ishidate et al. (1985)
<i>In vivo</i>				
Micronucleus	Mouse bone marrow (ddy mice, killed at 24 h)	1800 mg/kg bw once (intra-peritoneally) 1000 mg/kg bw four times over 24 h (intraperitoneally)	Negative Negative	Ishidate et al. (1988) ^b

^a In presence and absence of microsomal enzymes from Aroclor-induced rat liver (S9 mix)

^b Negative and positive control results were not shown in the English summary.

2.2.5 Special studies

(a) Effects on gastrointestinal microflora

In a study on the effects of pullulan on caecal microflora, 3-week-old male Sprague-Dawley rats were fed a diet containing 10% pullulan, equivalent to 5000 mg/kg bw per day, for 4 weeks. A control group received a diet containing 5% cellulose (equivalent to 2500 mg/kg bw per day). Food intake and body-weight gain were similar for the two groups, but faecal weight was significantly reduced in the group fed pullulan. When the caecal microflora were examined, the relative numbers of *Bifidobacteria* and *Streptococcus* were found to be increased and those of Bacteriodaceae decreased in comparison with controls (Sugawa-Katayama et al., 1994).

When six volunteers were given 10 g of pullulan (relative molecular mass, 50 000 Da) daily for 14 days, the substance was not detected in stool samples, and it was concluded that pullulan is completely fermented by intestinal bacteria. The faecal populations of *Bifidobacteria* increased in five of the six volunteers, from 11.9% to 21.9% (Yoneyama et al., 1990).

(b) *Aureobasidium pullulans*

A. pullulans is a ubiquitous, yeast-like fungus. It has been found in soil, on leaves, in lake water, on weathered wood, on latex paint films and synthetic plastic materials, as well as in used cosmetics and on foods such as fruits, cereals, tomatoes and cheese (Cooke, 1961; Durrell, 1967; Zabel & Terracina, 1980; Domsch et al., 1993; Mislivec et al., 1993; Vadkertiová & Sláviková, 1994; Weidenbömer et al., 1997; Webb et al., 1999; Cronin et al., 2000).

(i) *Pathogenicity*

In a study in rabbits, intramuscular injection of *A. pullulans* spores produced a nodule at the site of injection. No spread to other sites in the body was observed (Bulman & Stretton, 1974).

Intravenous injection of *A. pullulans* caused infection in the visceral organs of both healthy and immune-suppressed rats (Vishnoi et al., 2002).

A. pullulans has been isolated from humans but appears to occur as an opportunistic infection. *A. pullulans* was associated with fungal peritonitis in five patients receiving continuous ambulatory dialysis. It was also found in blood samples from a small number of immuno-compromised persons (Ajello, 1978; Kaczmarek et al., 1986; Salkin et al., 1986; Pritchard & Muir, 1987; Girardi et al., 1993).

(ii) *Toxicity*

Some strains of *A. pullulans* produce aureobasidin A, a cyclic depsipeptide that is toxic to fungi and yeast at low concentrations (0.1–0.5 µg/ml) but has low acute toxicity in mice ($LD_{50} > 200$ mg/kg bw) (Takesako et al., 1992). When the strain used for production of pullulan was analysed for aureobasidin A activity in *Saccharomyces cerevisiae*, none was detected (limit of detection, 2 ppm) (Hashimoto & Fukuda, 2002).

Two batches of pullulan were examined for the presence of the mycotoxins aflatoxin B₁, B₂, G₁ and G₂, zearalenone, sterigmatocystin and ochratoxin. None was found (Institut Européen de l'Environnement de Bordeaux, 2002).

The acute toxicity of *A. pullulans* in mice and rats is shown in Table 2.

In a study designed to assess the efficiency ratio of different microbial proteins, six male Long-Evans rats were fed a diet containing 27% *A. pullulans* cells (providing 12% crude protein in the diet) for 2 weeks. Their body-weight gain did not differ from that of a group fed a diet containing approximately 27% brewers' yeast. No signs of toxicity were observed (Han et al., 1976).

No signs of toxicity were observed in meadow voles (*Microtus canicaudus*) fed acid-hydrolysed straw that was subsequently fermented by *A. pullulans* for 10 days (Israelides et al., 1979).

(iii) *Allergenicity*

A. pullulans spores have been implicated in reactions such as allergic alveolitis and hypersensitivity pneumonia (Woodard et al., 1988; Karlsson-Borga et al., 1989; Kurup et al., 2000; Apostolakos et al., 2001); however, these allergic reactions have not been associated with ingestion of the vegetative form of the fungus. Moreover, there have been no reports over the past 25 years of allergic reactions in persons

Table 2. Acute toxicity of *Aurobasidium pullulans* administered orally

Species	Sex	No. animals/ group	LD ₅₀ (g/kg bw)	Reference
Mouse	Male	Not specified	> 24	Department of Public Hygiene (1974a) ^a
Rat	Male and female	5 per sex	> 20 (66.7% w/v <i>A. pullulans</i> lysate)	Ohnishi & Tsukamoto (1996)

^a No GLP or other guideline specified

exposed occupationally during fermentation of *A. pullulans* or production of pullulan (R. Asakura, personal communication, 2002).

2.3 Observations in humans

In a study of tolerance, 13 volunteers consumed 10 g of pullulan (relative molecular mass, 50 000 Da) daily for 14 days. Before and after pullulan intake, blood pressure and blood components (total, high-density and low-density lipoprotein cholesterol, β -lipoprotein, total fat, phospholipid, neutral fat, Ca, Na, K, Cl, aspartate and alanine aminotransferase activity and blood glucose) were measured in all volunteers. Faecal weight, pH, composition of faecal microflora and short-chain fatty acid concentration were examined in the faeces of six persons. No pullulan was detected in the stool samples, but daily stool weight was increased by 33%, and mean faecal pH was decreased in response to treatment (pH 6.5 before and pH 6.0 after pullulan intake). The faecal populations of *Bifidobacteria* increased in five of the six persons (11.9% total microflora before and 21.9% after treatment), and the short-chain fatty acids concentration increased from 6 to 8.8 mg/g faeces. No significant differences were observed in blood components. Abdominal fullness was the only symptom reported (Yoneyama et al., 1990).

In a study that addressed the effects of pullulan, dextran and soluble starch on bacterial flora, eight male volunteers received 10 g/day pullulan (relative molecular mass unspecified), dextran and soluble starch sequentially for 14 days with a 14-day wash-out period between each treatment. Before and after each 14-day treatment period, the men's faeces were tested for wet weight, relative change in wet weight, pH, total cell count per gram of fresh faeces, bifid bacteria ratio and relative change in bifid bacteria count. There was little difference in faecal bacterial count according to treatment. Pullulan and dextran resulted in increased faecal weight (from 129 g/day to 188 g/day with pullulan and 127 g/day to 144 g/day with dextran), and an increased percentage of bifid bacteria (12% to 25% with pullulan and 13% to 19% with dextran). No adverse effects were reported (Mitsuhashi et al., 1990).

3. DIETARY EXPOSURE

Pullulan is used as a substitute for gelatin in the production of capsule shells, as an ingredient of coated tablets and in edible flavoured films (breath fresheners). In Japan, it is used in a variety of foods, including savoury snacks, nuts and instant fried noodles, as a coating and glazing agent with oxygen barrier properties. Pullulan

is also widely used as an excipient in pharmaceutical tablets (Ministry of Health and Welfare, 1993).

No national assessments of exposure to pullulan were submitted. An estimated daily exposure based on data on the consumption of food supplements in the United Kingdom was submitted by the sponsor (Bär, 2004). The predicted exposures were based on the maximum use of pullulan in three products: capsule shells, tablets and flavoured films (Table 3).

Surveys in the United Kingdom on the consumption of food supplements indicated that 24% of 1724 adults, 14% of 1701 young persons (4–18 years) and 17% of 1675 toddlers (1.5–4.5 years) consumed food supplements (Gregory et al., 1995, 2000; Henderson et al., 2002). As no distinction was made between tablets and capsules, exposure was calculated conservatively, assuming that all supplements were in capsule form except those for toddlers. The 97.5th percentile of estimated exposure was seven capsules per day by adult consumers and two capsules per day by young people. Dietary supplements for children are usually formulated as tablets. If it is assumed that toddlers consume only tablets, with a consumption of seven tablets per day, the estimated 97.5th percentile exposure was 210 mg of pullulan per day. This hypothesis would result in ingestion of $\leq 135 \times 7 = 945$ mg/day pullulan for adults and $135 \times 2 = 270$ mg/day pullulan for young persons. The substitution of some or all of the capsules by tablets would result in lower exposure. The consumption of pullulan by children would be lower than that of adults and would typically not exceed 90 mg/day on the basis of three tablets per day (Bär, 2004).

Similar results were found for 259 adults in France during a survey of consumers of vitamin and mineral supplements (Touvier et al., 2004). Table 4 summarizes the number of capsules consumed by all persons and by consumers of capsules only.

A 'worst-case scenario' was proposed by the European Food Safety Authority (2004) Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food, which assumed that individuals would not normally take more than six food supplement capsules per day and that extreme consumers would not take more than double this amount ($135 \times 12 = 1620$ mg/day pullulan). In addition, the sponsor and the Panel assumed that persons would not consume more than one standard packet of breath-freshening films per day (700 mg pullulan). Therefore, the maximum daily exposure to pullulan for adults was estimated to be about 2.3 g for a person who ingested 12 supplements as capsules and a packet of pullulan strips per day. The actual exposure is likely to be lower. It was also assumed that small children would not consume this product.

Table 3. Estimated maximum levels of use of pullulan

Product	Amount of pullulan	Estimated maximum level of pullulan
Capsule shell (100–150 mg)	15–90%	135 mg per capsule
Tablet (1.2–1.5 g)	2% in tablet	30 mg per tablet
Pullulan-based flavoured film (32 mg per film)	$\leq 90\%$	29 mg per film 0.7 g per packet (12 films)

Table 4. Numbers of capsules taken per day at different percentiles of intake

Persons	Mean	Standard deviation	5th percentile	25th percentile	50th percentile	75th percentile	95th percentile
All	0.6	1.5	0.0	0.0	0.0	0.0	4.0
Consumers of capsules (21.7%)	2.6	2.3	0.4	0.9	2.0	4.0	7.0

Pullulan is used in Japan in various foodstuffs, at levels ranging from 2 g/kg in ham and sausages to 30 g/kg in processed products; a concentration of 50 g/kg was reported in hard sweets. A conservative estimate of dietary exposure from various foods was made with the budget method, assuming the presence of pullulan at the maximum reported level in a limited fraction of the diet (30 g/kg in 1/16 of the diet, corresponding to 187 g/day). This calculation resulted in a dietary exposure of about 6 g/day. Consumption of sweets by children was considered separately, with consumption figures available in France and the USA, resulting in an estimate of about 2.5 g/day.

5. COMMENTS

Toxicological data

Pullulan is largely resistant to digestion in the gastrointestinal tract as a result of the occasional presence of 1,3-glycosidic linkages and the high percentage of α -1,6-glycosidic linkages, which are resistant to hydrolysis by salivary and pancreatic amylases. The degree of digestion appears to depend on the relative molecular mass. A commercially available pullulan (relative molecular mass, 200 000 Da) releases only a small amount of reducing sugar after salivary amylase treatment but is converted to a substance with a lower relative molecular mass (about 70 000 Da) after treatment with an intestinal enzyme preparation.

Pullulan is fermented in the colon *in vitro* and *in vivo* by intestinal microflora, to produce short-chain fatty acids, although the degree of fermentation depends on the degree of polymerization of the pullulan. In humans, pullulan (relative molecular mass, 50 000 Da) could not be detected in faeces after daily consumption of 10 g for 14 days, suggesting that it was completely fermented. In contrast to maltodextrin, pullulan reduced the glycaemic response in healthy non-diabetic persons.

Although no studies were conducted to examine the effect of pullulan on the bioavailability of vitamins and minerals, there is no evidence from the published literature that similar polysaccharides of high relative molecular mass have adverse effects on vitamin or mineral bioavailability. When fed to rats at 20% in the diet, pullulan reduced intestinal calcium absorption but did not affect serum calcium levels.

The oral LD₅₀ of *A. pullulans* was reported to be > 24 g/kg bw. In rats, a single oral dose of *A. pullulans* lysate at 10 or 20 g/kg bw caused no signs of toxicity. Other studies indicate that *A. pullulans* does not produce toxins and is not toxic when fed to rats.

The oral LD₅₀ of pullulan was reported to be > 14 g/kg bw in mice. Short-term studies in rats showed that pullulan has little toxicity. In a 13-week study in rats given diets containing up to 10% pullulan (relative molecular mass, 200 000 Da), no evidence of treatment-related toxicity was found. The study showed a dose-dependent increase in caecum weight (full and empty) as a result of an increased level of poorly digested polysaccharide in the diet. This effect is considered to be a physiological response common to indigestible polysaccharides and of no toxicological significance. The NOEL was 10% in the diet, equal to 7900 mg/kg bw per day, on the basis of the highest dose used in this study. The results of other short-term studies in rats (9 and 62 weeks) support these conclusions. No long-term studies of toxicity or of reproductive toxicity were available on pullulan. Assays for genotoxicity with pullulan in vitro and in vivo assays gave negative results.

In a 14-day study in humans, daily consumption of 10 g of pullulan (relative molecular mass, 50 000 Da) had no adverse effects. The faecal *Bifidobacteria* population and short-chain fatty acid concentration increased, but no other clinical changes were observed. Abdominal fullness was the only clinical symptom reported. After a single dose of 50 g pullulan (relative molecular mass, 100 000 Da), the frequency of flatulence was increased for 24 h.

Assessment of dietary exposure

Pullulan is used as a substitute for gelatin in the production of capsule shells, as an ingredient of coated tablets and in edible, flavoured films (breath fresheners). The amount of pullulan ingested from one unit of each of these products is, respectively, 135 mg per capsule, 30 mg per tablet and 29 mg per film.

Specific data on consumption of food supplements were available from both France and the United Kingdom. For consumers at the 97.5th percentile, the intake of seven capsules per day was reported to correspond to a dietary exposure to pullulan of 950 mg/day. As dietary supplements for children are usually formulated as tablets, the consumption of pullulan by children was estimated to be lower than that of adults and typically not to exceed 90 mg/day on the basis of intake of three tablets per day, as reported in the United Kingdom. If a maximum daily consumption on a regular basis of seven capsules (950 mg/day of pullulan) and of one standard packet of breath-freshening films (700 mg/day of pullulan) is assumed, the maximum daily exposure to pullulan would be 1.65 g.

Pullulan is used in Japan in various foodstuffs, at levels ranging from 2 g/kg in ham and sausages to 30 g/kg in various processed products; use of 50 g/kg was reported in hard sweets. A conservative estimate of dietary exposure from various food by the budget method, assuming the presence of pullulan at the maximum reported level in a limited fraction of the diet (30 g/kg in 1/16 of the diet, corresponding to 187 g/day), resulted in a value of about 6 g/day. Consumption of sweets by children was considered separately, with consumption figures for France and the USA, resulting in an estimate of about 2.5 g/day.

The Committee recognized that the conservative estimates should not be summed.

6. EVALUATION

The Committee concluded that the current uses of pullulan as a food additive and the studies on its safety provided sufficient information to allocate an ADI 'not specified'.

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**Opinion of the Scientific Panel on Food Additives, Flavourings,
Processing Aids and Materials in Contact with Food
on a request from the Commission related to**

**Pullulan PI-20
for use as a new food additive**

Question number EFSA-Q-2003-138

adopted on 13 July 2004

SUMMARY

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food has been asked to evaluate pullulan PI-20 as a new food additive (foodstuffs in capsule and coated-tablet form) or as flavoured edible films (breath-freshening edible films).

Pullulan is a polysaccharide produced from a yeast. Pullulan has been used as a food ingredient for over 20 years in Japan. It has Generally Regarded As Safe (GRAS) status in the US for a much wider range of applications and thus higher intakes than the current application.

The proposed use is in the production of capsule shells and of coated tablets for the preparation of dietary supplements and as a matrix for edible flavoured films (breath fresheners). The toxicological database for pullulan is limited but indicates that pullulan is of low toxicity. Human volunteer studies have only reported abdominal fullness at doses of 10g pullulan per day with other mild gastrointestinal symptoms at higher doses. Exposure in adults at the specified worst case assumptions (12 tablets and a packet of breath freshening films) would be around 23% of this amount.

The Panel noted that the manufacturer claims a non-toxin producing strain of *Aureobasidium pullulans* is used for the production of PI-20, this should be included in the specification.

On the basis that pullulan is similar to other poorly digested carbohydrates and that the current proposed usage levels are below the level likely to cause abdominal fullness, the Panel

consider that the expected intakes of pullulan would not present any concern when used as a food additive in the proposed uses and at the usage levels requested. If higher levels of use or other uses were to be requested then more data might be required.

Key Words

Pullulan, food supplements, edible films, breath fresheners

BACKGROUND

A dossier for the use of pullulan as a new food additive was submitted to the Commission who subsequently asked EFSA to consider the safety of pullulan in the proposed uses. This work falls to the EFSA Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food.

Pullulan is a naturally occurring, fungal exopolysaccharide first described by Bender (1959). It has film-forming properties and can be used as a substitute for gelatine or other film-forming polymers in certain foods. The petitioner specifically addresses the use of pullulan in the production of gelatine-free capsules and coated tablets for dietary supplements and for edible flavoured films for consumption as breath fresheners.

Pullulan is produced by *Aureobasidium pullulans* a yeast-like fungus (Domsch *et al.*, 1993; Durell, 1967). *Aureobasidium pullulans* is also known as “black yeast” because it also produces melanin (Cooke, 1961)

According to the petitioner pullulan has been extensively used for more than twenty years in Japan where it is classified as a food ingredient. Its main use has been as a glazing agent with oxygen-barrier properties. Pullulan is accepted for use as an excipient in pharmaceutical tablets and is listed in the Japanese Standards for Ingredients for Drugs. It has GRAS status in the US for a much wider range of applications and thus higher intakes than those to be expected from the current application.

TERMS OF REFERENCE

The Commission asks the European Food Safety Authority to provide a scientific opinion on the safety of pullulan as a new food additive (foodstuffs in capsule and coated-tablet form) or as flavoured edible films (breath-freshening edible films).

ASSESSMENT

Technical data

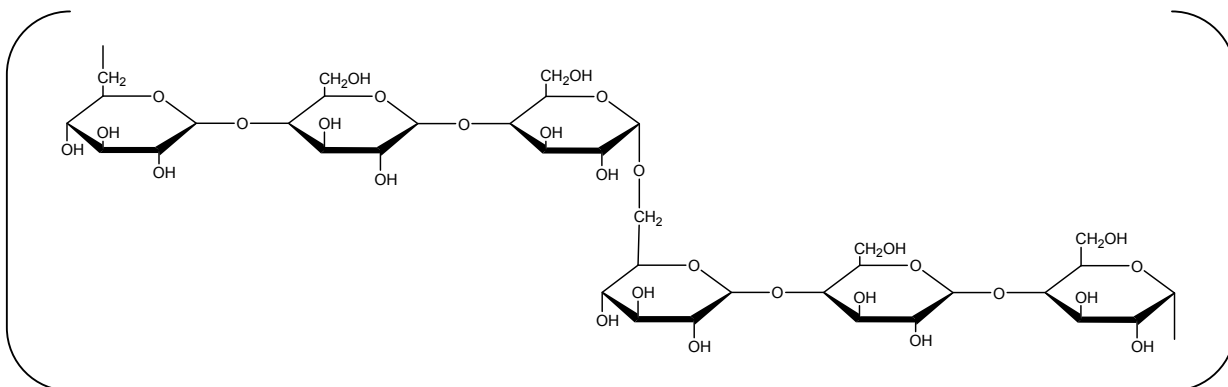
Pullulan is essentially a linear polymer of repeating maltotriose units consisting of two (1 → 4) linked α -D glucose molecules followed by a (1 → 6) linked α -D-glucose molecule. Maltotetraose units (Wallenfels, 1965, Carolan *et al.*, 1983) are often found at the terminal ends and may occasionally be found internally where they can be cleaved by salivary amylase which cleaves endo 1 → 4 glycosidic linkages (Catley *et al.*, 1986; Tsujisaka and Mitsuhashi, 1993). Occasional branch points (Wallenfels *et al.*, 1965) with 1 → 3 glycosidic linkages (Sowa *et al.*, 1963) may also be found (Tsujisaka and Mitsuhashi, 1993; Gibbs *et al.*, 1996).

The empirical formula of pullulan is $(C_6H_{10}O_5)_n$ where n routinely ranges from around 300 to 12,000 molecules i.e. molecular weights of approximately $0.05 - 2 \times 10^6$ daltons.

C.A.S. number 9057-02-7

EINECS number 232-945-1

Structural element of pullulan.



The molecular weight of pullulan can vary considerably (see above and numerous other references, e.g. Wallenfels *et al.* 1965, Wiley *et al.* (1993). Molecular weight standards are available from ~1000 daltons up (Okada *et al.* 1990).

The commercial product for which authorisation is requested is Pullulan PI-20 (P for pullulan, I to indicate that the product is deionised and 20 the average molecular weight of 200,000 daltons). According to the petitioner the product as commercialised (PI-20) “has a number average molecular weight (M_n) of about 100,000 to 200,000 daltons and a weight average molecular weight (M_w) of about 362,000 to 480,000 daltons (Okada *et al.*, 1990).

Production of pullulan by *Aureobasidium pullulans* occurs when the cells are in the late log phase (Catley, 1971) and stationary phase and is dependent on a range of factors including, temperature, pH, substrate, medium and strain (Catley, 1971, Gibbs and Seviour, 1996, Lazaridou *et al.*, 2002; Madi *et al.*, 1997, Sugimoto, 1978, Tsujisaka and Mitsuhashi, 1993, Ueda *et al.* 1963, 1996, Yuen, 1974). The yield and molecular weight of pullulan can be adjusted by manipulation of the substrate and fermentation conditions (Wiley, *et al.*, 1993).

Pullulan can be made into very thin films (down to 0.01 mm, Yuan, 1974). These have a high tensile strength and are stable over a range of temperatures. Pullulan films have a low oxygen permeability, are oil and grease resistant and dissolve rapidly in water. Pullulan films are usually prepared by rapid evaporation of a 5-10% aqueous pullulan solution applied to a smooth surface and dried; it may also involve the use of high temperature and pressure. Pullulan can also be made into shaped bodies. Optimally such bodies are made from pullulan of a molecular weight of around 250,000 daltons. This process usually involves rapid evaporation of water, compression moulding or extrusion at high temperature. A wide range of food, industrial and pharmaceutical applications have been cited for pullulan films. Pullulan can be mixed with a range of other food or non-food materials to alter the physical characteristics of pullulan films. Commonly pullulan may be mixed with gelatine, amylose and polyvinyl alcohol. Pullulan films or shaped bodies may also contain polyhydric alcohols as plasticisers; e.g. maltitol, sorbitol, glycerol and water soluble polyvinyl alcohol (Shih, 1996, Yuan, 1974, Biladeris *et al.*, 1999, Diab *et al.* 2001).

Description

A white to off-white tasteless, odourless powder that forms a viscous non-hygroscopic solution when dissolved in water at 5-10%. It can be made into films of high tensile strength and low oxygen permeability. Pullulan starts to decompose at 250°C and chars at 280°C (Tsujisaka and Mitsuhashi, 1993)

Solubility

Highly soluble in water, dilute alkali, insoluble in alcohol and other organic solvents except dimethylsulphoxide and formamide (Wallenfels *et al.* (1965). According to the petitioner a 10% w/w solution of PI-20 has a pH between 5 and 7.

Specifications

Proposed specifications for pullulan PI-20 have been provided by the petitioner. These are based on those detailed for pullulan listed in The Japanese Pharmaceutical Excipients (Ministry of Health and Welfare, 1993), which also includes a limit for heavy metals. These values are similar to those of some of the modified celluloses (JECFA, 1992; EC, 2003). According to the petitioner the substance is $\geq 90\%$ pure calculated as the difference between total dry weight and the sum of the known impurities (e.g. ash, mono, di and oligo saccharides). Details of impurities together with the method of analysis is provided by the petitioner. Possible impurities correspond to those listed in the proposed specifications, i.e. mono, di and oligosaccharides derived from the raw material (food grade corn starch) used to produce pullulan (not more than 10%), inorganic compounds (not more than 0.5%) and lead (not more than 1mg/kg). Additional specifications are used by the petitioner for internal quality control. These include limits for heavy metals, protein and arsenic. Levels of impurities as assessed from the analysis of 10 batches of PI-20 are included in the dossier. The suggested specifications were met for all batches. Independent analysis of 2 batches of pullulan for heavy metals, mycotoxins and microbiological contamination have also been provided. Analysis of three batches of pullulan PI-20 for mono, di and oligosaccharides indicated that an average of approximately 30% of the total (mono, di and oligosaccharides) had a degree of polymerization (DP) of 1, 27% had a $DP \geq 11$ (oligosaccharides) with the remainder being of intermediate sizes. According to the petitioner these non-pullulan carbohydrates are derived from the raw material used to produce pullulan (food grade corn starch).

Microbiological purity - Impurities could include the production strain, and cell wall components. Flat-sour bacteria spore-forming members of the genus *Bacillus*, (mainly *B. stearothermophilus* and *B. coagulans*) were detected in one batch out of two at 2 colony forming units/g analysed. These possibly originate from the raw materials. *A. pullulans* is removed from the fermentation broth by microfiltration and the filtrate sterilised by a proprietary heat treatment step using conditions sufficient to prevent carry over of the source organism (according to the petitioner *A. pullulans* is killed when heated to 60°C for 1 minute). *A. pullulans* was not detectable in 10 batches tested.

Production strain - The petitioner claims that their production strain has been selected by traditional means, without additional antibiotic resistance being introduced. It is not a genetically modified organism. The production strain has a high yield of pullulan, low production of melanin and does not produce aureobasidin A.

Viscosity

Pullulan solutions are viscous but do not gel. There is a linear relationship between the viscosity and molecular weight (Wallenfels *et al.*, 1965, Nakamura *et al.*, 1984). Viscosity is relatively independent of pH (<2 to >11) and temperature. Heating at 90°C for an hour reduces the viscosity of large polymers (around 300,000 daltons) by about 10% whereas there was little change in the molecular weight of smaller molecules (60,000–100,000 daltons). Viscosity is also unaffected by heating to 100°C for 6 hours in 30% NaCl (Sugimoto, 1978, Tsujisaka and Mitsuhashi, 1993).

Manufacture

Pullulan is made commercially by growing pullulan producing strains of *Aureobasidium pullulans* in appropriate media and then extracting and purifying pullulan from the culture media (Sugimoto, 1978, Yuen *et al.*, 1974). An overview of the production is given by Sugimoto (1978). Specific details of the manufacture of PI-20 by the petitioner are provided in the dossier. The following is an abbreviated version of the process taken from the petitioner's dossier. A non GM strain of *Aureobasidium pullulans*, selected for its high yield and low melanin production is used with corn syrup as a substrate. Following fermentation and

microfiltration to remove fungal cells the mixture is heat sterilised, decolourised, deionized and concentrated and then decolourised and filtered for a second time. The resulting filtrate is concentrated by evaporation to a nominal solid content, dried and crushed.

In-process controls are applied at all stages of the process. Particulate materials from the fungal cell wall are removed by microfiltration and treatment with activated carbon; ionic compounds (e.g. organic acids) are removed during the deionisation step; organic compounds (e.g. melanin, protein) get absorbed during the treatment with activated carbon; and volatile products (e.g. ethanol) volatilize during the final evaporation and drying.

METHODS OF ANALYSIS IN FOOD

As described by the petitioner this is a three step process involving;

- (a) isolation and extraction, which may be carried out either by Prosky's method (Prosky, *et al.*, 1988), or by aqueous extraction of the pullulan food and precipitation with methanol in the presence of KCl;
- (b) digestion with pullulanase;
- (c) analysis of samples of the initial extract from the first stage (a) and the pullulanase treated sample by high pressure liquid chromatography (HPLC) to determine concentrations of maltotriose molecules.

Case of need and proposed uses

The petitioner cites pullulan's film-forming properties as the basis for its proposed uses for the following:

- a substitute for gelatine, and thus suitable for vegetarians, in the production of capsule shells for dietary supplements;
- as an ingredient of coated tablets for dietary supplements; and
- as a matrix for edible flavoured films (breath fresheners).

Intended usage levels are 15 to 90% pullulan in capsule shells, up to 2% tablet weight when used as a coating for tablets and up to 90% pullulan in breath freshening films. Using the petitioner's estimate that the average capsule shell weighs 100 – 150 mg and that tablets weigh 1.2 – 1.5 g this equates to between 15 and 135 mg pullulan per capsule and 24 to 30 mg

pullulan per tablet. Breath freshening strips weigh 32 mg each so may contain up to 29 mg pullulan.

According to the petitioner the use of pullulan as a substitute for gelatine in coated capsules and tablets offers consumers, and especially vegetarians, the option of avoiding gelatine. In addition, the low oxygen permeability of pullulan films “protects susceptible ingredients (nutrients, colours, flavours) from deterioration and thus preserves the nutritional and organoleptic quality of the products”. According to the petitioner pullulan’s fast dissolving qualities and ability to act as a matrix for flavours makes pullulan based edible films suitable for use as instant breath fresheners that dissolve in the mouth.

Predicted exposure

The petitioner is unable to provide any data on the intake of dietary supplements in Europe nor does he provide an estimate of average consumption of breath-freshening edible films. An estimate of consumption was made by the Panel based on the assumption that individuals will not normally exceed six capsules per day and that extreme consumers will not take more than double this amount. Another assumption was that they would not consume more than a standard packet (containing 24 individual films) of breath freshening films per day. On this basis using the maximum usage levels, intake would be around 2.3 g pullulan per day. This assumes that an individual may ingest on a daily basis twelve supplements as capsules with 150 mg shells containing 90 % pullulan and the individual consumes a packet of breath freshening strips containing 90% pullulan.

UK data¹ on the consumption of food supplements provides information on the consumption of food supplements by adults (Henderson *et al.*, 2002) young persons, 4-18 years, (Gregory *et al.*, 2000) and toddlers aged 1.5 to 4.5 years, (Gregory *et al.*, 1995). The surveys indicate that 24% of adults², 14% of young people³ and 17% of toddlers⁴ consumed food supplements. These data do not discriminate between tablets and capsules, thus except for toddlers worst case intakes have been calculated assuming that all supplements were in capsule form. The

¹ Calculations provided by the UK Food Standards Agency

² based on 1724 adults surveyed

³ based on 1701 young people surveyed

97.5th percentile of estimated intake in consumers was 945 mg/day for adults (deriving from 7 capsules a day) and 270 mg/day for young people (deriving from 2 capsules a day). Assuming toddlers do not consume capsules the estimated 97.5th percentile intake in consumers was 210 mg pullulan per day (deriving from 7 tablets a day).

The panel considered that whereas small children probably do not consume breath fresheners this would not necessarily be true for older children and teenagers.

Information provided by the petitioner in support of its application for GRAS status as a food ingredient the US (see existing authorisations) and evaluated by the petitioner's expert panel quotes estimated average and 90th percentile daily intakes of pullulan as 9.4 and 18.8 g/person/day for the uses specified in its application for use as a food (which are wider than in the current request to the EU). The FDA considered these values in good agreement with their independent daily intake estimates of pullulan based on food categories and usage levels provided by the petitioner, which would be 10 g/person/day at the mean and 20 g/person/day at the 90th percentile (GRAS, 2002). The notification does not specify if these estimates are for the total population or consumers only.

Existing authorisations and evaluations

According to the petitioner pullulan has been used extensively in Japan for more than twenty years having been in commercial production since 1976. In Japan it is classified by the Food Chemical Section, Environmental Department, and Ministry of Health and Welfare as a food ingredient. It is also used as an excipient in pharmaceutical tablets and is listed in the Japanese Standards for Ingredients for Drugs (Ministry of Health and Welfare, 1993)

Pullulan was accepted for GRAS status in the US by the US Food and Drug Administration (FDA) in August 2002. This was based on the company's assertion that pullulan is GRAS (FDA, 2002) and supported by the report of an independent panel assembled by the petitioner. It examined the information on pullulan and considered it met GRAS criteria for its status as a food ingredient. The FDA's GRAS notice notes that pullulan has not been separately evaluated by the FDA and that the onus of ensuring that food ingredients marketed by the

⁴ based on a survey of 1675 toddlers

company are "safe, and are otherwise in compliance with all legal and regulatory requirements" lies with the manufacturer. The GRAS notice covers a wider range of applications than the current application to the EU Commission and thus higher pullulan intakes.

Microbiological Evaluation

Micro-organism

Aureobasidium pullulans (formerly *Pullularia pullulans*) is a non-pathogenic and non-toxic yeast-like fungus. It is commonly referred to as 'black yeast' due to melanin formation (Cooke 1961). The organism is ubiquitous. It is found in soil, lake water (Vadertiova 1994), weathered wood and plant leaves, on the surface of latex paint films (Zabel *et al.*, 1980) and synthetic plastic materials (Webb *et al.*, 1999), shared-use cosmetics (Mislivec *et al.*, 1993) and foods such as fruits, cereals, tomato and cheese. The petitioner claims that their production strain has been selected by traditional means, without additional antibiotic resistance being introduced. It is not a genetically modified organism. The production strain has a high yield of pullulan and low production of melanin. Some strains produce aureobasidin A, a cyclic depsipeptide which is toxic to fungi and yeast, but has an LD50 >200 mg/kg bw in mice. According to the petitioner no aureobasidin was detected from the production strain or culture medium filtrate using a sensitive yeast (*Saccharomyces cerevisiae*) tester strain (unpublished report, Takaharu Hasimoto and Shigeharu Fukuda, Amase Institute, Japan, 2002). No other mycotoxins have been detected in two batches analysed.

Effect on intestinal flora

Ingested pullulan is not significantly degraded by the digestive tract enzymes due to the high percentage (30%) of alpha 1,6-glucosidic linkages, whereas it was completely fermented by the microbial flora of the colon like other fermentable dietary fibre. Changes in caecal microbial flora were studied in S-D rats fed 10% pullulan, polydextrose and pectin. The relative number of bifidobacteria (in relation to total counts) was increased and *Bacteroides* decreased (Sugawa-Katayama *et al.*, 1994⁵). In human studies 10g of 50,000 dalton molecular weight pullulan ingested daily for 14 days was not detected in stool samples and therefore it was concluded that it was completely metabolised by the intestinal flora to short chain fatty acids.

The number of bifidobacteria increased from 11.9% to 21.9% of the total flora (Yoneyama *et al.*, 1990).

Absorption, distribution, metabolism and excretion

On the basis of the structure and molecular weight of pullulan, it can be assumed that it will not be absorbed as such.

In vitro studies

Human faecal cultures prepared from fresh stools (5 adult males) were incubated anaerobically with 4% w/w pullulan PR-5 (number average molecular weight 50,000) for up to 24 hours, then assayed for short chain fatty acids (SCFAs), water soluble saccharide and molecular weight of the undigested pullulan (Okada *et al.*, 1990). The pullulan was fully digested in 4 – 8 hours yielding a maximum of 52.7 g SCFA/100g pullulan. On this basis the energy value for SCFA was estimated as 2.05 kcal/g. The authors noted that assuming 100% SCFA absorption may be unrealistic and that with increasing pullulan intake more SCFA may be excreted in the faeces.

Digestion of pullulan film (molecular weight unspecified) and nine other polymers, including other carbohydrate polymers (starch - powder and film, levan and cellulose films) was investigated *in vitro* using an enzyme mix (Kunkel and Seo, 1994). A 1% pullulan solution was treated with the enzyme cocktail consisting of α -amylase, amyloglucosidase, peptidase, protease, invertase and lipase for up to 120 minutes. Analysis of oligosaccharides by HPLC provided an estimate of the degree of carbohydrate hydrolysis. Hydrolysis of pullulan was less than 10%. By comparison, hydrolysis of levan powder and cellulose film was less than 5%, whereas starch powder and starch film were hydrolysed by more than 90%.

Digestion of pullulan PI-20 (number average molecular weight around 200,000 daltons, containing about 8% low molecular weight - <10,000 dalton non pullulan sugars) and pullulan reagent PR-5 (a pullulan molecular weight standard, number average molecular weight 50,000, with no low molecular weight sugar contamination) were carried out using conditions designed

⁵ Paper in Japanese, abstract and figure legends in English, information from abstract

to simulate conditions in the human gut (Okada *et al.*, 1990). For PI-20 digestion was sequential with the mixture being sampled and desalted prior to proceeding to the next phase. Digestion was carried out using: (a) human saliva as a source of amylase, (b) artificial gastric juice (16.7 mM HCl-KCl, pH2.0), (c) commercial porcine pancreatic amylase and (d) a commercially available rat small intestinal enzyme preparation. The molecular weight of the hydrolysed pullulan was analysed by HPLC and showed that PI-20 was sequentially reduced in size to around 70,000 daltons following digestion with the rat intestinal enzyme preparation. PI-20 was cleaved by human saliva and pancreatic amylase without glucose release, but with a small increase in reducing sugar content (0.6 and 0.7% respectively). Digestion with the intestinal enzyme extract resulted in a 6.6% increase in glucose. PR-5 (50,000 daltons molecular weight) was hydrolysed by the rat intestinal enzyme preparation to produce 2.7% glucose but was not affected by any of the other treatments. The authors suggest that the glucose is produced by hydrolysis of the α 1→4 bond from the non-reducing end of the molecule and terminates at the α 1→6 bond. Pullulan standards with molecular weights ranging from 380,000 to 990 daltons were digested with small intestinal enzymes *in vitro* (as above). Above 100,000 daltons the amount of glucose released was 1.5% but increased with decreasing size to a maximum of 36% for the smallest sample.

Using their modification (Wolf *et al.*, 1999) of Muir and O’Dea’s (1992, 1993) validated *in vitro* protocol for mimicking physiological digestion conditions for “resistant starch” Wolf *et al.*, (2003), report that 95% of a pullulan preparation was hydrolysed in a five hour period compared to 0.5 hours for 98-100% hydrolysis of a maltodextose solution under the same conditions. They use these data to support their view that pullulan is a slowly digested carbohydrate (see human studies below).

Animal studies.

Fasted male Wistar rats (150-170g, 5 animals per group) were administered 2ml of a 10% pullulan (49,000 daltons, 302 glucose molecules) solution by gavage. Animals were killed 60 minutes later. Homogenates of the stomach and small intestine were analysed for glucose to estimate the extent of pullulan hydrolysis. Comparison of the glucose concentrations in homogenates of pullulan treated animals with those of control animals suggested that about 3% of the pullulan was hydrolysed, resulting in the release of glucose (measured as reducing sugar equivalents). The authors did not determine whether the pullulan hydrolysis products were absorbed in the small intestine. Data on the increase in reducing sugar obtained following

gavaging rats with a pullulan solution are close to the authors' theoretical estimate of approximately 2.5% hydrolysis of pullulan of this size calculated on the basis that 93% of the glucose molecules of the pullulan used in this study were maltotriose molecules and 7% maltotetrose molecules (which contain one alpha 1→4 linkage that would be susceptible to amylase, Cately *et al.*, 1986). However the authors also noted that the gut contains low level glycoamylase activity which is capable of hydrolysing alpha 1→4 and alpha 1→6 bonds from the non-reducing end (albeit very slowly) (Oku *et al.*, 1979).

Toxicology

Acute Toxicity

An oral LD50 of ≥14 g/kg was reported for pullulan (in olive oil suspension) in dd mice, based on 100% survival of animals given this dose. Similarly in a study using the same strain of mice the LD50 for *Aureobasidium pullans*, a pullulan producing yeast (suspended in distilled water) was reported as > 24 g/kg (Anon. a & b, 1974).

There were no signs of toxicity in groups of 5 male and 5 female Sprague Dawley rats (Crj: CD, SPF) in the 14 days following a single oral dose of a 66.7% lysate of *Aureobasidium pullans* at 10 or 20 g/kg bodyweight (Ohnishi and Tsukamoto 1996).

Sub-chronic Toxicity

Animal studies

Pullulan (molecular weight unspecified) supplied by the petitioner was administered in the diet to groups of fifteen 4 week old male and female Sprague Dawley rats (SD-JCL) for 62 weeks, when the study was terminated due to high mortality in all groups. Animals were fed *ad libitum* on a standard solid diet supplemented with 0, 1, 5 or 10 % pullulan, thus the diets were not isocaloric (Kotani *et al.* 1976, Kimoto *et al.* 1997). Animals were observed daily, body weights and food intake were recorded on a weekly basis. At termination internal organs were weighed and examined (macro and microscopically), blood and urine were taken for analysis.

Blood was tested for standard haematology: blood sugar, serum protein, albumin/globulin ratio, total cholesterol, serum transaminases (GOT, GTP), alkaline phosphatase. Urine was analysed for protein, sugar, blood, ketones and pH.

Mortality was high in all groups, and was reported to be due to pneumonia. Survival to termination showed a dose-related trend in females (87, 67, 67 and 40% at 0, 1, 5 and 10% pullulan, respectively) but not in males (47, 60, 27 and 47% at 0, 1, 5 and 10% pullulan, respectively). There were no significant differences in bodyweight gain or food consumption. For male rats food consumption ranged from between 123 and 144 g/kg bw/day at week one to 34 – 41 g/kg bw/day at week 62; average intake of control animals over the 62 weeks was 46 g/kg bw/day. Food intake for females was 110 to 132 (week 1) to 42 to 47 g/kg bw/day (week 62), average intake of the control animals over the 62 week period was 56 g/kg bw/day. Average pullulan intakes over the 62 week period for males were: 4.4 g/kg bw/day pullulan for the 10% group; 2.3 g/kg bw/day for the 5% group and 0.5 g/kg bw/day for the 1% group. Similarly the average pullulan intakes for females over the same period were: 5.2, 2.6, and 0.5 g/kg bw/day for the 10%, 5 and 1% pullulan fed groups. The terminal bodyweights of the low and high dose males were significantly lower than those of the controls, but this effect was not dose-related (89, 96 and 91% of control at 1, 5 and 10% pullulan, respectively). There were no significant differences in the females. Some significant differences were observed in absolute organ weights. In males the absolute weight of brain was increased (low dose), absolute weights of heart, liver, kidney and submandibular gland were decreased. In females, the absolute weight of the brain was decreased (low and mid dose), absolute weights of heart, liver, caecum and spleen were increased at some doses. Since these changes were not clearly dose-related and relative organ weights did not differ significantly, they are unlikely to be of biological relevance. The authors suggested that the 46% increase in absolute caecal weight in the females treated with 10% pullulan was a physiological response to undigested pullulan. A small number of statistically significant changes were observed in haematological and clinical chemistry parameters, which were not dose-related. No significant differences were observed in urine analysis for any of the groups. Reported histopathological observations did not suggest any dose-related effects but confirmed that animals from all groups had bronchitis. This study indicates that 62 weeks administration of pullulan at dietary concentrations up to 10%, (4.4g/kg bw day for males and 5.2g/kg bw day for females) did not result in adverse effects. However, the value of the study is limited by the infection and poor survival.

Pullulan (Molecular weight 49,000 daltons, circa 302 glucose molecules) was administered in the diet to groups of 5-10 male Wistar rats (50-60g) in two separate studies (Oku *et al.*, 1979). In the first study doses of 0, 20 and 40% pullulan (approximately 10 and 20 g pullulan/kg bw/day) were fed for 4 or 9 weeks. In the second study doses of 0, 5 and 10% pullulan (approximately 2.5 and 5 g pullulan/kg bw/day) were fed for 4 or 7 weeks (pullulan) or 4 or 9 weeks (control). The control and pullulan diets contained 4% cellulose with a total carbohydrate content of 69%, achieved by addition of cornstarch. Bodyweights were recorded at unspecified intervals throughout the studies. At termination, internal organs were weighed. The authors noted that “several” rats in the 40% pullulan groups occasionally developed diarrhoea or soft faeces throughout the study period, whereas rats in other dose groups did not. Pullulan administration resulted in a dose-related decrease in bodyweight gain, with bodyweights significantly different from control in the 20 and 40% dose groups from about 10 days until the end of the study at 9 weeks (terminal bodyweights 86 and 78% of control, respectively). At 5 and 10% pullulan, bodyweights were significantly reduced after 10-14 days, but not at the end of the study (7 weeks).

After 4 weeks of treatment, the relative stomach weights were significantly increased (to about 120% of control) at 5 and 10% pullulan, but not at 20 or 40% pullulan. This pattern was apparently reversed by longer administration, with increased relative stomach weight (112% of control) after 9 weeks at 20 and 40% pullulan and no change after 7 weeks at 5 and 10% pullulan. The relative small intestine weights were increased at 20 and 40% pullulan after 4 weeks (140 and 160% of control, respectively), and by about 20% at all doses at the longer time points. The relative large intestine weights were increased after 4 weeks at all doses of pullulan (130-169% of control), and at the two higher doses at the longer time points (about 130% of control). Relative caecal weights were also significantly increased at the 20 and 40% pullulan (4 weeks: 251 and 279%; 9 weeks: 202 and 212% of control, respectively). The authors considered that the differences between 4 weeks and 7/9 weeks were indicative of physiological adaptation. However, it should be noted that there were no control animals sacrificed at the 7 week time point for direct comparison with the 5 and 10% pullulan treated animals. The authors reported that there were no changes in the weights of other organs (“liver, spleen, heart, kidney, adrenal glands, lung, brain, etc.”) but no data were presented.

Intestinal mucosal homogenates prepared from the 9 week pullulan treated animals from the above study showed no significant differences in the activity of intestinal maltase, sucrase or isomaltase compared to homogenates from control animals (Oku, 1979).

Six week old male Sprague-Dawley rats (8 animals per group) were fed diets containing 1% and 10% pullulan (no details on specification) for 4 weeks and compared to a control group on a diet containing 5% cellulose (Sugawa-Katayama *et al.*, 1993)⁶. Carbohydrate content was normalised to 68% with corn starch, thus the diets were not isocaloric. The colon mucosae were analysed for cell size, by comparing protein to DNA ratios, and by scanning electron microscopy. Statistically significant increases were observed in the wet weight of the colon mucosa in the 10% pullulan fed group. Mucosal protein content was decreased in the pullulan fed rats, with a greater effect at 1% than at 10%. DNA content was significantly increased in the 10% pullulan fed group. The authors suggested that these data indicated that pullulan decreased the size of the colon mucosal cells. Faecal weight was decreased by pullulan in a dose-related manner. Scanning electron microscopy of colons suggested that the haustra coli were broader than the control in pullulan fed animals (Sugawa-Katayama *et al.*, 1993)⁷.

Intestinal calcium absorption has been reported as being reduced following the consumption of large doses of non-digestible carbohydrate (Reinhold *et al.*, 1976 and reviewed by Gordon *et al.*, 1995). Pullulan (molecular weight unspecified) was administered to male Wistar rats (40-50 g, 6/group) at 20% in the diet for 8 weeks. Diets were normalised to a total carbohydrate content of 67% with corn starch and were therefore not isocaloric. Animals were fasted for 16 hours before sacrifice and then a partially purified duodenal homogenate was prepared. Calcium binding (measured by competitive binding with a cation exchange resin) in the duodenal supernatant was significantly reduced compared to control (64% of control). Alkaline phosphatase activity was reduced by about 50% and sucrase by about one third⁸. The authors suggested that these data were indications of mechanical damage to the mucosal surfaces resulting in loss of calcium binding protein and enzyme leakage from the mucosal cells (Oku *et al.*, 1982).

Mutagenicity

⁶ abstract and figures in English, text in Japanese

⁷ abstract and figures in English, text in Japanese

Information on mutagenicity testing of pullulan is limited, with inadequate experimental detail and no information on the specification of the tested material. However, considering the structure and molecular weight of pullulan, genotoxicity is not expected.

Pullulan at 10 to 10,000 µg per plate did not increase numbers of revertants in *S. typhimurium* strains TA1535, TA100, TA1537 and TA 98 with and without S-9 activation in a plate incorporation assay (Anon 1978, Kimoto *et al.*, 1997).

No firm conclusion on genotoxicity can be drawn from tests on differential toxicity in *B. subtilis* strains (Hachiya⁹ *et al.* 1985, Kuroda *et al.* 1989¹⁰).

Pullulan did not induce chromosome aberrations in Chinese hamster cells (CHL strain) at concentrations up to 12 mg/ml with 24 and 48 hour harvest times (Ishidate *et al.*, 1985)¹¹.

Pullulan was negative in a mouse micronucleus assay when administered by intraperitoneal injection at 1800 mg/kg once or 1000 mg/kg 4 times over a 24 hour period to groups of 6 male mice (ICR strain CD1-mice) aged about 8 weeks old (Ishidate *et al.*, 1988)¹².

Carcinogenicity, Reproductive and Developmental Toxicity.

No data available

Other Studies

Human volunteer studies

Thirteen male volunteers (24 –53 years, average age of 34.5), were given 10g (approximately 0.17g/kg bw assuming a 60 kg individual) of reagent grade PR–5 pullulan (50,000 dalton

⁸ based on histograms

⁹ Figures in English, text in Japanese

¹⁰ Abstract and figures in English, text in Japanese

¹¹ Paper in Japanese, short English extract provided

¹² details of positive controls were not provided in the short English translation

molecular weight) daily for 14 days. Pullulan powder was dissolved in soup or water and given to volunteers at lunchtime each day. Apart from a request not to consume excessive alcohol no restrictions were placed on the volunteers. Six of the 13 volunteers (average age 32, range 24 -53) provided complete stool samples on the morning of the first dose of pullulan and on the morning after the 14 days of pullulan consumption. Stools were also collected and weighed for the 48 hours prior to pullulan intake and for the final 48 hours of pullulan intake. Faecal pH, SCFA concentration, levels of water soluble saccharides and faecal microflora were analysed. All 13 volunteers had their blood pressure measured together with a range of blood tests (total, HDL and LDL cholesterol, β -lipoprotein, total fat, phospholipid, neutral fat, Ca, Na, K, Cl, GOT, GTP and blood glucose). There was no treatment related change in stool frequency among volunteers. Post abdominal fullness after consuming pullulan was reported in “some” of the volunteers. No other effects were reported. There was an apparent decrease in faecal pH and increase in faecal SCFA content in 5/6 test subjects after treatment with pullulan, but these were not statistically significant. Water soluble saccharide content and blood test results were within the normal range. There was no significant difference in the total number of faecal micro-organisms per g faeces before and after pullulan intake, but pullulan appeared to alter the spectrum of bacterial flora in a number of individuals, the most marked difference being an increase in *Bifidobacteria* in 5/6 volunteers. Pullulan was not detected in the faeces. The authors suggested that pullulan was metabolised to SCFAs by the intestinal micro-organisms in the large intestine and that most of the SCFAs are absorbed by the intestinal tract (Yoneyama *et al.*, 1990).

Another volunteer study investigated the effects of pullulan, dextran and soluble starch on bacterial flora in human volunteers (Mitsuhashi *et al.*, 1990). Eight male volunteers (average age 33.4 years, weight 62.8 kg) were treated sequentially with 10 g/day (0.16 g/kg bw/day) pullulan (molecular weight unspecified), dextran or soluble starch I in soup for 14 days at lunch time. There was a 14 day wash out period between each treatment so that each individual acted as his own control. On a per gm basis there was little overall change in faecal bacterial count with any of the treatments used. Administration of both pullulan and dextran resulted in an increase in faeces weight and percentage bifidobacteria after 14 days treatment. Faecal wet weights increased from 129 ± 30 g/day to 188 ± 35 g/day (146%) with pullulan and from 127 ± 28 g/day to 144 ± 30 g/day with dextran (113%). The equivalent percentage of

bifidobacteria as a proportion of the total bacterial cell count was approximately doubled for pullulan (24.8 compared to 12.0).

A number of other studies have addressed the issue of whether pullulan affects blood glucose levels.

Wolf *et al.* (2003) investigated the digestibility and glycemic effect of pullulan in non-diabetic, healthy male and female volunteers (average weight 73.4 kg, range 53 - 105). Nineteen male and nine female volunteers took part in a randomised double blind two treatment, two period crossover meal tolerance tests. Volunteers were asked to consume a high carbohydrate diet for 3 days prior to the experiment, and avoid exercise for the 24 hours prior to dosing. Individuals were randomly assigned to treatment groups and on the evening before a “treatment day” they were given a low residue liquid food and solid energy bar meal designed to provide a third of their daily energy requirement X 1.3. After an overnight fast a blood sample (pin prick) was obtained for blood glucose measurement prior to the administration of a sterilised flavoured drink containing 50g pullulan (100,000 daltons molecular weight) or 50g maltodextrin. Following the meal blood glucose was measured every 15 minutes for the first 60 minutes and then every 30 minutes up to for 180 minutes. In addition a breath hydrogen analysis was carried out to evaluate carbohydrate malabsorption and volunteers were asked to report any symptoms due to ingestion for the two 24 hour periods following ingestion of the pullulan meal. The crossover experiment was carried out 5 to 13 days after the first meal. Postprandial blood glucose concentrations were reduced in individuals consuming the pullulan based drink when compared to those consuming maltodextrin. Pullulan increased carbohydrate malabsorption and led to increased intolerance symptoms compared to the maltodextrin [largely flatulence which was more common in the first 24 hour post-prandial period (22 ± 6 of 28 individuals compared to 5 ± 3) than the second (8 ± 3 of 28 compared to 4 ± 2)]. Time to peak glucose concentration is delayed in the pullulan treated individuals, with a significant difference ($P < 0.0001$) in the incremental AUC of 135 mmol min/L for pullulan compared to 268 mmol min/L for maltodextrin. The authors conclude that their *in vivo* and *in vitro* data show that pullulan is slowly digested in the human gut and leading to a broader flatter rise in blood glucose compared with maltodextrose.

Hiji (1990) reported that a 1:20 to 1:400 pullulan to starch or sucrose ratio reduced peak blood glucose levels in man though there was considerable variation in the required dosage

depending on a range of factors including the pullulan molecular weight, the age and health of the individual. (The same patent reports similar results in animal studies). In another study a 39 year old human volunteer was given a solution of 50 g glucose plus 0, 5 or 10 g pullulan in 200 ml water. Blood glucose was monitored every 30 minutes for 180 minutes. The addition of pullulan to the glucose solution had no effect on blood glucose levels that were near identical with all three treatments (Oku¹³ *et al.*, 1983).

Allergenicity and Immunogenicity

Allergic alveolitis and hypersensitivity pneumonia has been linked to inhalation of *A. pullulan* spores and not the vegetative form. The petitioner claims that other fungi which are equally allergenic have been safely used for the production of food or food enzymes for decades. There are a number of reports of environmental exposure to *Aureobasidium pullulans*, the source organism for pullulan production, leading to respiratory symptoms including hypersensitivity pneumonitis (Woodard *et al.*, 1988; Apostolakos *et al.*, 2001) and other lesions (Ajello, 1978). The fungus is also reported to be a frequent source of respiratory allergy (Kurup *et al.*, 2000) and IgE antibodies to *Aureobasidium pullulans* have been identified in sera from individuals with suspected mould allergy (Karlsson-Borga *et al.*, 1989). Experimentally it has been demonstrated to cause extrinsic allergic alveolitis in the rabbit (Bulman, 1974).

Aureobasidium pullulans was identified as being associated with fungal peritonitis in a small number of patients undergoing continuous ambulatory peritoneal dialysis. Pritchard and Muir (1987) screened 556 dematiaceous hyphomycetes (black fungi that include *Aureobasidium pullulans*) received in their laboratory over a five year period. Thirty five isolates were considered to be of “probable pathogenic significance”. Five hundred and fourteen isolates (2/3 of which were *Aureobasidium pullulans*) were considered “unlikely” to be of pathogenic significance (the remaining 7 (none *Aureobasidium pullulans*) of “possible pathogenic significance”

Opportunistic infection with *Aureobasidium pullulans* has been reported in immunocompromised patients by Salkin *et al.* (1986), Kaczmariski *et al.* (1986) and Giradi *et al.* (1993).

¹³ Paper in Japanese, abstract and figure legends in English.

Over a 25 year period there have been no occurrences of allergic reaction to *A. pullulans* amongst workers at the production site (letter provided by a physician from the petitioner's company clinic, March 26, 2002).

There do not appear to be any specific data on the allergenicity of ingested pullulan.

DISCUSSION

Usage levels have been proposed for pullulan as a new food additive used in the production of capsule and tablet shells as a substitute for gelatine and as a flavour matrix in breath freshening films. An estimate of consumption was based on these usage levels and the assumption that individuals will not normally exceed 6 capsules per day and that extreme consumers will not take more than double this amount. Another assumption was that they would not consume more than a standard packet (containing 24 individual films) of breath freshening films per day. On this basis the maximum intake would be around 2.3 g pullulan per day. This assumes all supplements are taken as capsules with 150 mg shells containing 90 % pullulan and the individual consumes a packet of breath freshening strips containing 90% pullulan. This intake may be considered a worse case intake for adults, teenagers and older children.

The Panel noted that the manufacturer claims a non-toxin producing strain of *Aureobasidium pullulans* is used for the production of PI-20, this should be included in the specification

The toxicological database is limited. The pullulan product under consideration is PI-20, with an average molecular weight of 200,000 daltons. Many of the available studies provided no information on the type of pullulan used. Of those that did, the majority used a material with a molecular weight of about 50,000 daltons. *In vitro* studies suggest that PI-20 is broken down into smaller polymers (of around 70, 000 daltons) by salivary and pancreatic amylases (Okada *et al.* 1990). *In vitro* and *in vivo* experiments suggest that it may be fermented to short chain fatty acids in the colon. It is assumed to be completely fermented but *in vivo* evidence for this is unclear and there are no data to indicate whether the rate of fermentation depends on the size of the polymer. No adequate chronic toxicity studies are available nor are there data on carcinogenicity, reproductive toxicity or developmental toxicity. Subchronic (9-62 week) studies in the rat indicate that pullulan is of low toxicity. Pullulan is a soluble carbohydrate

polymer that is poorly digested by intestinal enzymes. Studies in which pullulan was administered in the diet to rats for up to 9 weeks suggest that pullulan has local effects in the gastrointestinal tract but provided no evidence of systemic effects. Increased relative weights of the stomach, small intestine, large intestine and caecum and evidence of changes in the size and shape of intestinal pouches (the haustra coli) in the intestinal mucosa were reported at dietary concentrations of 1% pullulan (around 0.5 g pullulan/kg bw/day) and greater. Limited evidence indicated a decrease in severity of effects with time of administration, suggesting possible adaptation.

Human volunteer studies have reported mild gastrointestinal symptoms at doses of 10g pullulan per day and greater (i.e. approximately 0.17 g/kg bw/day for a 60 kg individual). At 10g the only reported gastrointestinal symptom was abdominal fullness. The estimated exposure in adults using the specified worst case assumptions (12 tablets and a packet of breath freshening films) would be around 23% of this amount. If the same worse case assumptions were applied to children weighing 30 kg, exposure expressed per kg body weight would be 46% of this amount.

Pullulan has similarities to a number of other poorly digestible carbohydrate polymers including modified celluloses. In 1992 the Scientific Committee on Food (SCF) reviewed 5 modified celluloses. The SCF noted that modified celluloses are practically non-absorbed, are of low toxicity and do not possess carcinogenic properties. The SCF considered “the observed gastro-intestinal effects in feeding studies were related to the physical effects of the bulk and hydrophilic properties of the material” and traditional toxicological evaluation procedures were not considered to be appropriate (SCF, 1994; 1999).

CONCLUSIONS AND RECOMMENDATIONS.

The Panel noted that the manufacturer claims a non-toxin producing strain of *Aureobasidium pullulans* is used for the production of PI-20, this should be included in the specification.

On the basis that pullulan is similar to other poorly digested carbohydrates and that the current proposed usage levels are below the level likely to cause abdominal fullness, the Panel consider that the expected intakes of pullulan would not present any concern when used as a

food additive in the proposed uses and at the usage levels requested. If higher levels of use or other uses were to be requested then more data might be required.

DOCUMENTATION PROVIDED TO EFSA

Dossier prepared and submitted on behalf of the petitioner (Hayashibara) for the evaluation of pullulan as a new food additive pursuant to Council Directive 89/107/EEC (Bioresco, 2002) 2 annexes containing a number of documents and publications (not all the papers and articles supplied have been referenced in the text as they include several reviews and material that is not relevant to the safety assessment).

Additional information, papers on immunogenicity and an unpublished report on a skin sensitisation test have been provided by the petitioner, May 2004

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ACKNOWLEDGEMENT

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food wishes to thank Caroline Tahourdin for her contribution to the draft opinion.

File No PLC/693

May 2007

**NATIONAL INDUSTRIAL CHEMICALS NOTIFICATION AND ASSESSMENT SCHEME
(NICNAS)**

FULL PUBLIC REPORT

Pullulan

This Assessment has been compiled in accordance with the provisions of the *Industrial Chemicals (Notification and Assessment) Act 1989* (Cwlth) (the Act) and Regulations. This legislation is an Act of the Commonwealth of Australia. The National Industrial Chemicals Notification and Assessment Scheme (NICNAS) is administered by the Department of Health and Ageing, and conducts the risk assessment for public health and occupational health and safety. The assessment of environmental risk is conducted by the Department of the Environment and Water Resources.

For the purposes of subsection 78(1) of the Act, this Full Public Report may be inspected at our NICNAS office by appointment only at 334-336 Illawarra Road, Marrickville NSW 2204.

This Full Public Report is also available for viewing and downloading from the NICNAS website or available on request, free of charge, by contacting NICNAS. For requests and enquiries please contact the NICNAS Administration Coordinator at:

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**Director
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FULL PUBLIC REPORT**Pullulan****1. APPLICANT AND NOTIFICATION DETAILS**

APPLICANT(S)

Hayashibara International Australia Pty Ltd (ABN 61 120 127 488)
Level 31, ABN AMRO Tower
88 Phillip St
Sydney NSW 2000

NOTIFICATION CATEGORY

Polymer of Low Concern

EXEMPT INFORMATION (SECTION 75 OF THE ACT)

No details are claimed exempt from publication.

VARIATION OF DATA REQUIREMENTS (SECTION 24 OF THE ACT)

No variation to the schedule of data requirements is claimed.

PREVIOUS NOTIFICATION IN AUSTRALIA BY APPLICANT(S)

None

NOTIFICATION IN OTHER COUNTRIES

US FDA (2002)
US TSCA (2001)
Canada (1998)
EU (1990)
European Food Safety Authority (2006)
Korea (1997)

2. IDENTITY OF CHEMICAL

CHEMICAL NAME

Pullulan

OTHER NAME(S)

1,4-2,6-alpha-D-glucan
1,6-alpha-linked maltotriose

MARKETING NAME(S)

Pullulan (INCI Name)
Pullulan PI-20

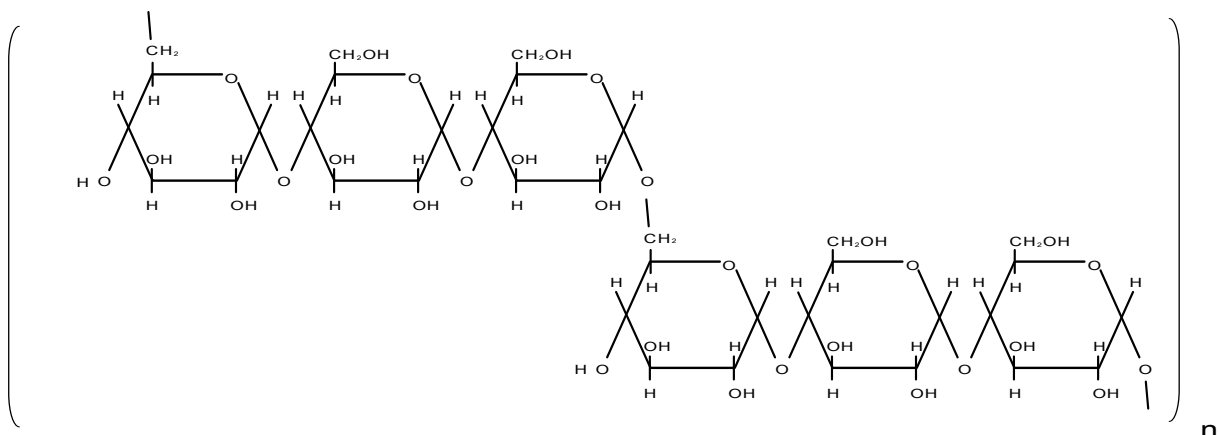
CAS NUMBER

9057-02-7

MOLECULAR FORMULA

Unspecified

STRUCTURAL FORMULA



where n = approximately 210

MOLECULAR WEIGHT

Number Average Molecular Weight (Mn)	97,000-101,000
Weight Average Molecular Weight (Mw)	433,000-479,000
Polydispersity Index (Mw/Mn)	4.3-4.9
% of Low MW Species < 1000	1.2-2.4 (consists of oligosaccharides with 3-7 glucose subunits)
% of Low MW Species < 500	0

POLYMER CONSTITUENTS

Pullulan is a fungal polysaccharide. It is produced on an industrial scale by fermentation of food grade corn syrup under controlled conditions using a specific, not genetically modified strain of *Aureobasidium pullulans*, a ubiquitous, non-pathogenic and non-toxigenic yeast-like fungus. The raw material (corn syrup) consists of a number of saccharides including glucose, fructose and maltose. The amount of residual mono and di-saccharides is typically 1.5-3.4%. There was no evidence for the presence of *Aureobasidium pullulans* in ten examined batches of pullulan.

REACTIVE FUNCTIONAL GROUPS

The notified polymer contains only low concern functional groups.

3. PLC CRITERIA JUSTIFICATION

Criterion	Criterion met (yes/no/not applicable)
Molecular Weight Requirements	Yes
Functional Group Equivalent Weight (FGEW) Requirements	Yes
Low Charge Density	Yes
Approved Elements Only	Yes
Stable Under Normal Conditions of Use	Yes
Not Water Absorbing	Yes
Not a Hazard Substance or Dangerous Good	Yes

The notified polymer meets the PLC criteria.

4. PHYSICAL AND CHEMICAL PROPERTIES

Appearance at 20°C and 101.3 kPa	White to off-white free flowing powder
Melting Point/Glass Transition Temp	Decomposes at 250°C
Density	280 kg/m ³ at 25°C
Water Solubility	≥ 170 g/L at 20±5°C The test substance was vigorously shaken for 30 seconds at 5 minute intervals for 30 minutes. The viscosity of the resulting solution prevented testing of water solubility at higher concentrations.
Particle Size	Inhalable (< 100 µm): 7.1% Determined by a sieving method. No values given for MMAD or respirable fraction (<10 µm).
Reactivity	Stable under normal conditions of use.
Degradation Products	None under normal conditions of use. Self ignites at 250°C to give carbon oxides.

5. INTRODUCTION AND USE INFORMATION

MAXIMUM INTRODUCTION VOLUME OF NOTIFIED CHEMICAL (100%) OVER NEXT 5 YEARS

<i>Year</i>	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	<i>5</i>
<i>Tonnes</i>	0.5	0.5	0.5	0.5	0.5

USE AND MODE OF INTRODUCTION AND DISPOSAL

Mode of Introduction

The notified polymer will be imported as a powder in 10 kg polyethylene bags with rubber seals inside cardboard boxes. The notified polymer will be transported by road to the notifier's warehouse, dedicated to the storage of chemical products, before being transported to customer sites (cosmetic formulators). The notified polymer may also be imported as part of preformed articles (up to 30% notified polymer).

Reformulation/manufacture processes

The notified polymer will not be manufactured in Australia.

Cosmetics

Reformulation is anticipated to occur at a number of cosmetics manufacturing sites. This will involve blending the notified polymer with other ingredients, and will not involve reaction of the notified polymer.

In a typical process, a compounder will weigh out the notified polymer manually. This will then be manually added to a mixing tank, along with other ingredients. During the blending a chemist may take samples of the product containing the notified polymer (concentration up to 20%) using a dip tube. After the blending is complete a packer will supervise the use of a line filler and capper to transfer the finished product into the retail bottles. The packaged cosmetic products will then be stored and handled by a store person.

Biodegradable articles

There are currently no plans to manufacture biodegradable articles containing the notified polymer in Australia.

Use

The notified polymer is used in biodegradable articles (concentration up to 30%), or in cosmetic products, such as:

- Shampoos (< 4%)
- Creams and lotions (< 1%)
- Styling products (as an impermeable, antistatic solid film) (< 10%)
- Toothpastes (< 20%)

6. HUMAN HEALTH IMPLICATIONS**6.1. Exposure Assessment****OCCUPATIONAL EXPOSURE***Transport and storage*

Transport and warehousing workers are expected to have dermal and ocular contact with the notified polymer and products containing the notified polymer only in the event of accidental spillages. Inhalation exposure to the polymer powder may also occur if the packaging is breached.

Reformulation

Dermal, ocular and inhalation exposure to the notified polymer powder may occur during weighing out prior to reformulation. However, exposure to significant amounts of the notified polymer is typically reduced by the use of ventilation at the site of weighing, such as a dust extract hood, or by the use of a vacuum tube. The compounder is also expected to wear personal protective equipment such as glasses, gloves and coveralls.

Dermal and ocular exposure to the polymer solutions may also potentially occur during certain processes involving the notified polymer such as sampling, cleaning, maintenance, or by accidental spills during the packing process. However, exposure to significant amounts of the notified polymer is limited because of the largely automated processes, and the engineering controls and personal protective equipment worn by workers.

Beauty Industry

Intermittent, wide-dispersive use with direct handling is expected to occur among hairdressers, cosmeticians, and beauticians. According to EASE (1997) modelling of this work environment, dermal exposure in the range of 1-5 mg/cm²/day of products containing up to 10% of the notified polymer (assuming maximum concentration from cosmetic/styling products) could result. Assuming 100% dermal absorption, a surface area of 420 cm² (half the area of the hands) and a bodyweight of 60 kg, this equates to a maximum systemic exposure of 3.5 mg/kg bw/day.

Retail Industry

Workers in the retail industry will only be exposed to the notified polymer (up to 20%) in the event of packaging breaches or accidental spillages.

PUBLIC EXPOSURE*Biodegradable Articles*

The notified polymer will not be sold to the public except in the form of finished articles. There are no specific details on the types of articles, or their uses. However one possibility is trays comprised partly of the notified polymer. Although there is potential for extensive public exposure to these articles, blooming/leeching of the notified polymer from the articles is not expected and hence exposure to the notified polymer is considered to be low.

Cosmetics

Since the notified polymer will be in products sold to the general public, widespread public exposure is expected. Exposure to the notified chemical will vary depending on the type of cosmetic product and individual use patterns. Based on exposure estimates for a range of cosmetic products in Europe (SDA,

2005), public exposure (dermal and oral) to the notified polymer in Australia has been estimated using the following assumptions:

- Bodyweight of 60 kg;
- 100% dermal and oral absorption
- Product usage is similar in Australia to Europe.

<i>Product used and exposure type</i>	<i>Amount of product used per day (g/day)</i>	<i>Product retained/ingested (%)</i>	<i>Concentration in product (%)</i>	<i>Exposure (mg/kg bw/day)</i>
Skin lotions (dermal)	5.68	100	< 1	0.95
Shampoos (dermal)	8	1	< 4	0.05
Styling products (dermal)	10	5	< 10	0.83
Toothpastes (oral)	2.4	35	< 20	2.8
Range of cosmetic products (dermal and oral)				4.63

The estimate of combined exposure to a range of cosmetic products (skin lotion, shampoo, styling product and toothpaste), 4.63 mg/kg bw/day, is expected to be an overestimate as it assumes all products used by one person contain the notified polymer and uses the maximum 'product amount used' from the range in the dataset.

Since products containing the notified chemical are stored and used in a domestic environment, there is the possibility of accidental ingestion by a child.

6.2. Toxicological Hazard Characterisation

The notified polymer meets the PLC criteria and can therefore be considered to be of low hazard. This is supported by toxicological endpoints observed in testing conducted on the notified polymer. Only studies for which reports were provided are summarised in the table below.

<i>Endpoint</i>	<i>Result</i>	<i>Classified?</i>	<i>Effects Observed?</i>	<i>Test Guideline</i>
Skin irritation – human patch test	non-irritating at 25%	no	no	In-house method
Eye irritation, HET-CAM* test	no ocular irritation potential in vivo predicted at 100%	no	no	In-house method
Eye irritation, Bovine corneal opacity and permeability test	no negative effect at 20% compared to the control	no	no	In-house method
Skin sensitisation – human repeat insult patch test	no evidence of sensitisation or irritation at 25%	no	no	In-house method
Rat, oral repeat dose toxicity – 13 weeks (notified polymer in feed)	NOAEL: 7914 mg/kg bw/day (males); and 9674 mg/kg bw/day (females)	no	yes	OECD TG 407
Genotoxicity - bacterial reverse mutation	non mutagenic	no	no	In-house method

* HET-CAM: Hen's egg test-chorio allantoic membrane.

Although the *in vitro* eye irritation tests and human patch tests are not validated for classification, the negative results in all tests indicate low hazard of the notified polymer.

In a 13 week repeat dose study in which the notified polymer was given to the rats in the feed, no adverse effects were observed. Test item-related macroscopic changes were restricted to a dose-

dependently increased caecum weight and a distension of the caecum at necropsy in 1/10 males in the low dose group, 3/10 males in the mid dose group and 1/10 males in the high dose group. Since no microscopic changes were seen in the affected caeca these findings were not considered to be adverse. The NOAEL was therefore determined from the dose given to the high dose group (10% in feed).

No mutagenicity was observed in the strains *Salmonella typhimurium* TA1535, TA100, TA1537 or TA98 both with and without metabolic activation when tested with the notified polymer up to 10 mg/plate.

All results were indicative of low hazard. In addition, although no studies were cited the results for testing on the notified polymer are reported as:

- no adverse treatment related effects in a 62 week repeat dose study when treated at a maximum dose of 4500 mg/kg bw/day (males) / 5100 mg/kg bw/day (females);
- negative in a DNA repair test;
- negative in an in vitro chromosomal aberration assay;
- negative in an in vivo mouse micronucleus test.

The notified polymer is a soluble polymer with molecular weight > 13,000, and may therefore have the potential to cause lung overloading effects due to decreased clearance from the lung.

6.3. Human Health Risk Assessment

OCCUPATIONAL HEALTH AND SAFETY

Although limited exposure to the notified polymer could occur during reformulation processes and during use in the beauty industry, the risk to workers is considered to be low due to the intrinsic low hazard of the notified polymer. The maximum dermal exposure for workers involved in the beauty industry is estimated to be 3.5 mg/kg bw/day. A dermal NOAEL was not determined, however a lowest NOAEL of 7914 mg/kg bw/day was established in a 90-day feed study in the rat. The use of this NOAEL results in a margin of exposure (MOE) of 2261. MOE greater than or equal to 100 are considered acceptable to account for intra- and inter-species differences. In addition the MOE is based on conservative assumptions and may overestimate the risk.

Given the low percentage of inhalable/respirable particles and the typical engineering controls in place, the risk of lung overloading effects is considered to be low. However, the level of atmospheric nuisance dust should be maintained as low as possible. The NOHSC exposure standard for atmospheric dust is 10 mg/m³.

PUBLIC HEALTH

Members of the public may make dermal contact with biodegradable products containing the notified polymer. However, the risk to public health will be negligible because the notified polymer is of low hazard, and is bound within a matrix.

The public will be exposed to the notified polymer during use of cosmetic and personal care products. The combined exposure to a range of cosmetic and personal care products is estimated to be 4.63 mg/kg bw/day. A dermal NOAEL was not determined, however a lowest NOAEL of 7914 mg/kg bw/day was established in a 90-day feed study in the rat. The use of this NOAEL results in a margin of exposure (MOE) of 1709. MOE greater than or equal to 100 are considered acceptable to account for intra- and inter-species differences. In addition the MOE is based on conservative assumptions and may overestimate the risk. In addition the risk of local effects after use of product containing the notified polymer is considered to be low due to the predicted low hazard of the notified polymer.

7. ENVIRONMENTAL IMPLICATIONS

7.1. Exposure Assessment

ENVIRONMENTAL RELEASE

The notified polymer is not manufactured in Australia but will be reformulated into cosmetic consumer products at customer sites. As the polymer is solid only a small quantity (<0.1%; < 0.5 kg per annum) is expected to remain in import packaging. Up to 3% (< 15 kg per annum) is expected to remain in blending tanks during reformulation. This will be flushed to sewer. It is expected that approximately 1% (< 5 kg per annum) of the formulated product will remain in the consumer product packaging. The remainder is expected to be used as intended, in consumer cosmetic products and will be eventually flushed to sewer.

ENVIRONMENTAL FATE

No biodegradation test was submitted, but two studies were provided which show that relatively rapid biodegradation is expected. The polysaccharide polymer is expected to degrade to simple sugars then to oxides of carbon and water vapour. The residue in packaging, and biodegradable articles containing the notified polymer, are expected to be sent to landfill where they will degrade. Similarly, the notified chemical is expected to degrade in the sewage treatment plant or in natural waterways after release.

7.2. Environmental Hazard Characterisation

No ecotoxicological data were submitted. PLCs without significant ionic functionality are of low concern to the aquatic environment.

7.3. Environmental Risk Assessment

The predicted environmental concentration (PEC) for a worst case scenario, where 99% of the polymer is flushed to sewer, throughout Australia without degradation or adsorption to sludge is calculated as 0.33 µg/L (495 kg per annum ÷ (200 L per person per day × 365 days × 20.5 million persons)). A predicted no effect concentration (PNEC) cannot be calculated, but PLCs without significant ionic functionality are of low concern to the aquatic environment. Although a risk quotient (RQ) cannot be calculated from the PEC/PNEC ratio, there will be an adequate safety margin and the risk to the aquatic environment is expected to be acceptable. Furthermore the notified polymer is expected to degrade by biotic and abiotic processes, thus further reducing the risk to the environment.

8. CONCLUSIONS

8.1. Level of Concern for Occupational Health and Safety

There is Low Concern to occupational health and safety under the conditions of the occupational settings described.

8.2. Level of Concern for Public Health

There is No Significant Concern to public health when used in the proposed manner.

8.3. Level of Concern for the Environment

The polymer is not considered to pose a risk to the environment based on its reported use pattern.

9. MATERIAL SAFETY DATA SHEET

9.1. Material Safety Data Sheet

The notifier has provided MSDS as part of the notification statement. The accuracy of the information on the MSDS remains the responsibility of the applicant.

10. RECOMMENDATIONS

CONTROL MEASURES

Occupational Health and Safety

- No specific engineering controls, work practices or personal protective equipment are required for the safe use of the notified polymer itself, however, these should be selected on the basis of all ingredients in the formulation.

Guidance in selection of personal protective equipment can be obtained from Australian, Australian/New Zealand or other approved standards.

- A copy of the MSDS should be easily accessible to employees.
- If products and mixtures containing the notified polymer are classified as hazardous to health in accordance with the NOHSC *Approved Criteria for Classifying Hazardous Substances*, workplace practices and control procedures consistent with provisions of State and Territory hazardous substances legislation must be in operation.

Disposal

- The notified polymer should be disposed of by authorised landfill.

Emergency procedures

- Spills and/or accidental release of the notified polymer should be handled by removing possible leaking containers and sweeping up spills for reuse to the extent practicable or disposal. Wash the area with water.

10.1. Secondary Notification

The Director of Chemicals Notification and Assessment must be notified in writing within 28 days by the notifier, other importer or manufacturer:

- (1) Under subsection 64(1) of the Act; if
 - the notified polymer is introduced in a chemical form that does not meet the PLC criteria.or
- (2) Under subsection 64(2) of the Act:
 - if any of the circumstances listed in the subsection arise.

The Director will then decide whether secondary notification is required.

Specifications

PULLULAN JP Pullulan

Definition: Pullulan is a neutral simple polysaccharide produced by the growth of *Aureobasidium pullulans*. It has a chain structure of repeated α -1,6 binding of maltotriose composed of three glucoses in α -1,4 binding.

Storage: Store at 1 - 30°C in a dark and dry place.

Shelf Life: Twelve (12) months from the production date when stored unopened

Package: Polyethylene bag in a carton box (Net 10 kg)

Compatible Specification: Japanese Pharmacopoeia

Variables	Specifications	Test Method
Identification	Positive for Pullulan only	JP Monograph
Viscosity (10% solution)	120-140 mm ² /s (at the time of manufacture)	JP Monograph
pH	4.5 – 6.5	JP Monograph
Loss on drying	Not more than 6.0%	JP Monograph
Residue on ignition	Not more than 0.3%	JP Monograph
Mono-oligosaccharide	Not more than 1.0%	JP Monograph
Monosaccharide, disaccharide and oligosaccharides	Not more than 10.0%	JP Monograph
Heavy metals (as Pb)	Not more than 5ppm	JP Monograph
Nitrogen	Not more than 0.05%	JP Monograph
Cadmium	Not more than 0.5ppm	ICP-AES
Arsenic	Not more than 1.5ppm	JP General test method
Lead	Not more than 1.0ppm	ICP-AES
Mercury	Not more than 0.1ppm	ICP-AES
Abs. at 270nm (10% solution)	Not more than 0.500	JP General test method
Total aerobic microbial count	Not more than 100 CFU/g	JP General test method
Total Yeast and mold count	Maximum 50 CFU/g	JP General test method
<i>Escherichia coli</i>	Absence in 1 gram	JP General test method
<i>Salmonella</i>	Absence in 10 grams	JP General test method
<i>Staphylococcus aureus</i>	Absence in 1 gram	JP General test method

JP: The Japanese Pharmacopoeia



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1. Identification of the Substance and the Company

Identification of the substance

Product Name : PULLULAN

Company identification

Manufacturer

Company Name : Hayashibara Co., Ltd.

Address : 578 Imabo, Kita-ku, Okayama 701-0145, JAPAN

Contact in Emergency

Company Name : Hayashibara Co., Ltd.

Address : Nihon-Seimei Okayama Bldg. II Shinkan
1-1-3 Shimoishii, Kita-ku, Okayama 700-0907, JAPAN

Responsible Department : Sales & Marketing Center, Business Planning Office

Telephone : +81-86-224-4312 (9:00 a.m. – 5:30 p.m. Japan time)

Fax : +81-86-233-2265

General Use : Food ingredient / Food additive

2. Hazards Identification

GHS Classification

Physical Hazards : Not applicable

Others : Not applicable, Not classified or Classification not possible.

Symbol : No symbol

Signal Word : No signal word

Hazard Statement : No statement

Precautionary Statements : No precautionary phrases

Other Hazards which do not result in classification : None

3. Composition / Information on Ingredients

Substance/Preparation : Substance

Purity : Not less than 90%

Chemical Identity : Poly[6)- α -D-glucopyranosyl-(1 \rightarrow 4)- α -D-glucopyranosyl-(1 \rightarrow 4)- α -D-glucopyranosyl-(1 \rightarrow)]

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Chemical Formula : (C₁₈H₃₀O₁₅)_n
CAS No. : 9057-02-7

4. First Aid Measures

General Information : Not expected to be a health hazard when used under normal conditions.

Inhalation : Remove to fresh air and keep at rest in a position comfortable for breathing.

Skin Contact : Immediately take off all contaminated clothing. Wash areas thoroughly with water.

Eye Contact : Immediately flush eyes with a sufficient amount of water. Remove contact lenses if easy to do so. Continue rinsing.

Ingestion : If material is swallowed in large amounts, get medical attention.

Consult a doctor, if symptoms develop or persist after taking the measures above.

5. Fire Fighting Measures

Clear fire area of all non-emergency personnel.

Extinguish Media : Dry chemical, foam, carbon dioxide, water fog

Unsuitable Extinguishing Media : No information

Special Fire Fighting : Position upwind. Keep unnecessary personnel away. Move containers out of hazard area if safe to do so. Keep the containers cool by spraying water if exposed to heat or fire. Cool containers with flooding quantities of water until well after until well after the fire is out.

Protection for Fire Fighter : Wear self-contained breathing apparatus and protective clothing to prevent contact with skin and eyes.

6. Accidental Release Measures

Avoid contact with spilled or released material. See Chapter 13 for information on disposal. Observe the relevant local and international regulations.

Personal Precautions : Use personal protection recommended in Chapter 8. Avoid breathing mist and contact with skin and eyes.

Environment Procedures : Prevent entry into ditches, sewers and waterways.

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Containment and Cleanup : Sweep up the spill and dispose of in general trash. Wash residual with water. Spill area can be washed with water for approved disposal. Follow all regulatory requirements for non-hazardous waste disposal.

7. Handling and Storage

Handling : Use in a well ventilated place. If necessary, use personal protection recommended in Chapter 8. Wash thoroughly after handling. When handling, do not eat, drink or smoke. Coloration and decomposition may occur by moisture absorption.

Storage : Keep container tightly closed. Keep in a cool, dark and dry place in order to maintain the quality. Keep securely closed when not in use. Keep away from food, drink and animal feeding stuffs.

8. Exposure Controls / Personal Protection

Exposure Limit Value : Not set.

Exposure Controls : No specific controls are needed. If necessary, the following personal protective equipments and materials are applied.

Personal Protective Equipments and Materials (if necessary)

Respiratory Protection : If ventilation is not sufficient to effectively remove and prevent buildup of dusts, appropriate respiratory protection should be provided.

Hand Protection : Wear impervious gloves for prolonged contact.

Eye/Face Protection : Wear appropriate eye protection.

Skin Protection : Wear impervious apron and/or boots for skin protection in case scattering.

9. Physical & Chemical Properties

Appearance : White to light yellowish white powder

Odor : Odorless or slight characteristic odor

pH : 5.0 - 7.0

Melting Point : No data available

Boiling Point : No data available

Flash Point : No data available

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Evaporation Rate	: No data available
Flammability	: No data available
Explosive Properties	: Dust Explosion Risk Minimum (dust cloud) Ignition Temperature ("MIT", °C) 420 Layer (5 mm layer) Ignition Temperature ("LIT", °C) > 400
Vapor Pressure	: No data available
Vapor Density	: No data available
Specific Gravity	: No data available
Solubility	: No data available
Partition Coefficient	: No data available
Relative Self-ignition	: No data available
Decomposition Temperature	: No data available
Viscosity	: 100 - 180 mm ² /S

10. Stability and Reactivity

Chemical Stability	: Stable under ordinary storage conditions.
Possibility of Hazardous Reactions	: Not likely to occur.
Conditions to Avoid	: Avoid accumulation of airborne dusts. Avoid hot and humid storage.
Materials to Avoid	: Avoid contacting strong oxidizing agents, which may react with the product.
Hazardous Decomposition Products	: In case of fire, the product emits carbon monoxide, carbon dioxide and/or low molecule weight hydrocarbons.

11. Toxicological Information

Acute Oral Toxicity	: LD ₅₀ ≥ 14,280 mg/kg (male dd mice, oral) LD ₅₀ ≥ 24,134 mg/kg (male RF mice, oral)
Skin Irritation/sensitization	: No data available [Reference data: No dermal irritation (Human, 25%, RIPT)]
Eye Damage/Irritation	: Not cause any negative effect (BCOP, 30%) Practically no irritation (HET-CAM, 100%)
Germ Cell Mutagenicity	: No mutagenicity (mouse micronucleus and chromosome aberration assays) [Reference data: Not mutagenicity (AMES test)]

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Carcinogenicity	: No data available
Reproductive Toxicity	: No data available
STOT-Single Exposure	: No data available
STOT- Repeated Exposure	: No data available
Aspiration Hazard	: No data available

12. Ecological Information

Toxicity	: No data available
Persistence & Degradability	: No data available
Bioaccumulative Potential	: No data available
Mobility in Soil	: No data available
Other Adverse Effects	: No data available

13. Disposal Considerations

Comply with each local regulation.

Follow all regulatory requirements for non-hazardous waste disposal when dump this material into sewers, on the ground or into any body of water.

14. Transport Information

UN Transport Hazard Class(es)	: Not classified
UN Number	: None
UN Proper Shipping Name	: None
Environmental Hazards	: None
Special Precautions for Users	: Confirm no leakage from the container. Embark the shipment without any upset, dropping and damage, and prevent collapse of the shipment robustly. Comply with each local regulation.

15. Regulatory Information

The product is not subject to classification according to the sources of literature known to us.

Please refer to national measures that may be relevant.

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16. Other Information

This information is furnished without warranty, express or implied, except that it is accurate to the best knowledge of Hayashibara Co., Ltd. It relates only to the specific material designated herein, and does not relate to use in combination with any other material or in any process. Hayashibara Co., Ltd. assumes no legal responsibility for use of or reliance upon this information.

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Appendix G

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NAGASE Group

November 14, 2017

To: Capsugel

Subject: JP PULLULAN LMO / Packaging Information

Hayashibara Co., Ltd. confirms that the inner polyethylene bag used for JP PULLULAN LMO (LMOP) complies with the food packaging regulations stipulated in the Japanese Food Sanitation Law.

The low density polyethylene film is used for the bag of LMOP and contacts with LMOP. The test result of this film shows that it meets the Standards and Criteria for Food and Food Additives, the Food Sanitation Law (Announcement No. 370 issued by the Ministry of Health and Welfare in 1959).

<u>Test Item</u>	<u>Specifications</u>	<u>Result</u>
Material Test		
Lead	not more than 100 ppm	comply
Cadmium	not more than 100 ppm	comply
Migration Test		
Heavy metals	not more than 1 ppm (as Pb)	comply
Consumption of KMnO4	not more than 10 ppm	comply
Residue on evaporation		
Water	not more than 30 ppm	comply
4% acetic acid	not more than 30 ppm	comply
20% alcohol	not more than 30 ppm	comply
n-heptan	not more than 150 ppm	comply

If you have any question, please feel free to contact us through our distributor.

Sincerely,

Makoto Kikkawa
Division Manager
Quality Assurance Division

The information in this document is based on current knowledge and experience of Hayashibara Co., Ltd., however, it is provided as general information without any obligation or assumption of liability. Because use conditions and applicable laws may differ from one location to another and may change with time, the information is used at your own discretion and risk.

HAYASHIBARA CO., LTD.

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<http://www.hayashibara.co.jp/en>

November 14, 2017

To: Capsugel

JP PULLULAN LMO / 10kg package

Package	Japanese side	English side
		
Printed Lot number	Japanese side	English side
		
Inner Package and Label		
Lot numbering system	<p>Example for Lot No. 5K1024</p> <ul style="list-style-type: none"> 5 : Last digit of manufacture year K : Month of manufacture (A to L : 1 to 12) 10 : Date of manufacture 24 : Specific number <p>Lot No. 5K1024 shows that this lot is manufactured on November 10, 2015.</p>	

Makoto Kikkawa

Makoto Kikkawa
Division Manager
Quality Assurance Division

HAYASHIBARA CO., LTD.

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Example of Pullulan Capsules, Ready for Shipment



Plantcaps™

The more natural choice.

Introducing **Plantcaps™** capsules, the more natural alternative for the savvy green consumer.



Plantcaps capsules from Capsugel are the new premium capsules designed for the needs of the growing and very discerning healthy lifestyle market. Globally, more than 50 percent of consumers are willing to pay a premium price for a natural product, and more than 48 percent say they're willing to pay more for organic products.¹ In the U.S., 35 percent of supplement users say that vegetarian or non-animal source is important when choosing a supplement, up from 26 percent in 2006—and this group is among the most frequent users of supplements.² In a recent study of supplement users in Europe, more than 45 percent said they would be more likely to purchase a supplement if they knew it was in a vegetarian, plant-based capsule.³



Today's healthy lifestyle consumer is savvy. Not content to take labels at face value, they investigate everything they put into their bodies, especially their supplements. They seek "real" ingredients—with no artificial chemicals, pesticides or preservatives. They want to be certain that each ingredient is safe and proven, because they're conscientious about maintaining their health. This commitment to a green lifestyle extends beyond diet and nutrition and into every aspect of their lives. They want products that have the attributes that fit their lifestyle, but they don't wish to sacrifice quality or performance.

New Plantcaps capsules help protect ingredients and healthy lifestyles.

Plantcaps capsules provide the premier balance of performance and purity for healthy lifestyle consumers who are looking for naturally healthy attributes in the products they buy. Made from tapioca which is naturally fermented into **pullulan**, a starch-free vegetarian capsule, Plantcaps capsules speak to the needs of the most discerning consumer. Plantcaps capsules provide all of the benefits of gelatin capsules while still meeting the dietary needs of vegetarian and vegetarian-aware supplement users. They deliver the product claims that healthy lifestyle customers look for when making purchase decisions, like starch-free, gluten-free, preservative-free, vegetal origin and non-GMO. They are the more natural alternative and they meet dietary needs, with GRAS status, Halal and Kosher certifications.

Plantcaps capsules are everything your green consumers want in a capsule, with nothing they don't.

A plant-based capsule made from tapioca created through natural fermentation

- Proven in the food and pharmaceutical markets for more than 25 years

Meets the needs of consumers' healthy lifestyles

- Organic labeling allowed in the U.S. when filled with organic ingredients*
- A vegetarian capsule that meets important product claims: starch-free, gluten-free, preservative-free, vegetable origin, non-GMO
- Meets dietary needs, certifications include GRAS status, Halal and Kosher
- Helps mask taste and smell
- Beautiful, crystal-clear transparency, with high luster
- Manufactured in accordance to cGMP guidelines, with a certified quality assurance system for traceability of raw materials
- Allowed for use in Japan

Provides ease of formulation

- Disintegration/dissolution profile equal to gelatin
- The best oxygen barrier properties of any vegetarian capsule material
- Non-reactive shell can help eliminate "spotting" concerns, works well with Vitamin C
- New patent-pending formulation provides robust performance with a wide range of ingredients

Proven filling performance

- Highly stable; proven under accelerated aging conditions
- Excellent machinability on all capsule filling machines, equivalent to gelatin capsules
- Compatible with wide range of excipients



KEY PERFORMANCE CLAIMS

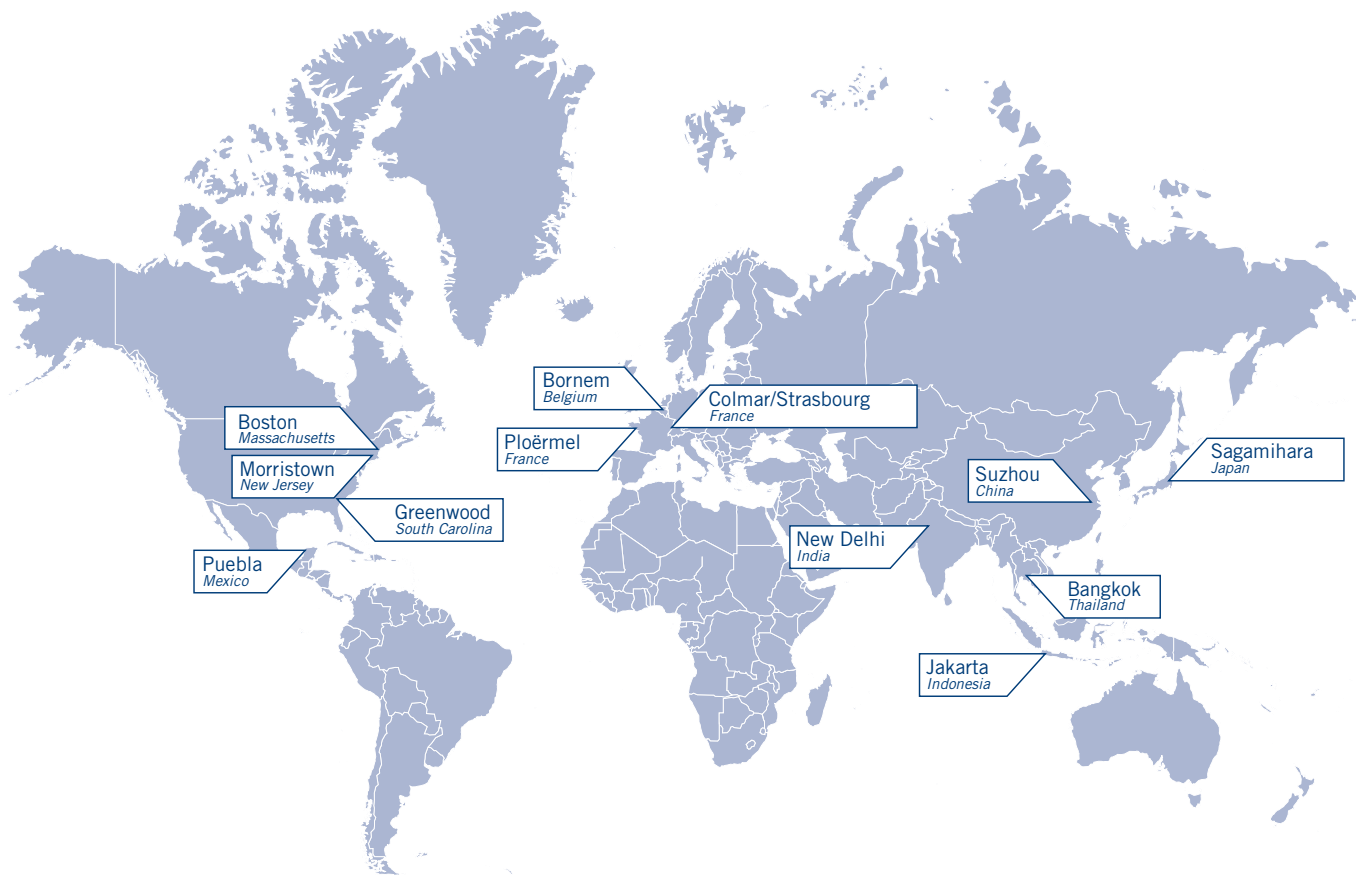
- » All the benefits of gelatin in a vegetarian capsule
- » Organic label language allowed in the U.S. when filled with organic ingredients*
- » The more natural alternative, starch-free, gluten-free, preservative-free, vegetable origin and non-GMO
- » The best oxygen barrier properties
- » Suitable for Asian regulatory environments

*Contact your organic certifying provider for further details.

¹Euroonitor Annual Study 2011: Green Influences – interactive survey of 16,000 respondents globally

²Natural Marketing Institute, Supplement/OTC/Rx Database (SORD) Overview, November 2011

³Capsugel Non-Animal Portfolio Survey, US, UK, Germany, France & Italy, February 2012



Success Requires More Than Just a Great Capsule.

With a commitment to innovation, Capsugel offers:

- Extensive global supply capability
- State-of-the-art process controls
- Formulation support, technical support and filling/sealing equipment
- ISO, GMP, Kosher and Vegetarian certifications

For more information contact us at marketing.amer@capsugel.com.

CAPSUGEL®