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February 26, 2004

Capsugel Division of Pfizer Inc.
535 North Emerald Road
Greenwood, SC 29646

National Organic Standards Board
C/o Arthur Neal, Agriculture Marketing Specialist
STOP 0268 – Room 4008-S
1400 Independence Avenue, SW.
Washington, D.C. 20250-0200

PETITION FOR LISTING OF PULLAN ON THE USDA NATIONAL LIST OF ALLOWED AND PROHIBITED SUBSTANCES

The Organic Foods Production Act of 1990, as amended, established a National List of Allowed and Prohibited Substances National List) which identifies the synthetic substances that may be used, and the no synthetic substances that cannot be used, in organic production and handling operations. The Act also provides a mechanism to petition the National Organic Standards Board to evaluate a substance for inclusion on or removal from the National List. With this petition, Capsugel request review of the pullulan (dry basis) for consideration and, if appropriate, listing on the Proposed National List of Organic substances for inclusion on:

- Nonagricultural (organic) substances allowed in or on processed products labeled as "organic" or "made with organic (specified ingredients)."

ISO 9002 SGS

Capsugel Regulatory Affairs

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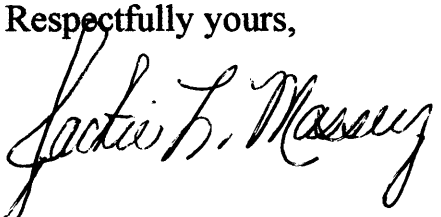
Capsugel appreciate the time and effort that the Department of Agriculture, Agricultural Marketing Services, invests in the review of petitions for organic status. Please feel free to contact me by phone or e-mail address as listed below, if you have any questions on any aspect of this petition.

Respectfully yours,



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Petition for the Inclusion of NPCaps™ on the National Organic Standards Board List of Approved Organic Substances

Based on relevant regulatory requirements, the component Pullulan (dry basis) is qualitatively acceptable for use in pharmaceuticals and dietary supplements in Japan, the US, Canada, Australia, and the EU. Furthermore, no safety issues were identified in authoritative reviews of the Pullulan (dry basis) that would raise a concern for it use. However, pharmaceutical and dietary supplement products using Pullulan (dry basis) should be assessed within the context of the final pharmaceutical/dietary supplement formulation [including the active ingredient(s)] and anticipated exposure levels.

Item A:

With this petition, Capsugel Division of Pfizer is requesting the evaluation of Pullulan (dry basis) for inclusion on the National List:

- Nonagricultural (nonorganic) substances allowed in or on processed products labeled as "organic or "made with organic (specified ingredients)."

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Item B

1. Substance Name: Pullulan (polysaccharide), dry basis

   Chemical Name. Poly-alpha-1, 6-(alpha-1, 4-maltoriose)

   Synonyms. Pururan

   CAS Number. 9057-02-7

   Empirical formula. (C6H10O5) n where n= 300 to 3000

   Functional Category. Multiple technical effects as a food additive

   Regulatory Citations. Pullulan (No. 373) is an existing food additive permitted for general food use (included in the list of food additives from natural origin complied and published by the Ministry of Health and Welfare on April 16, 1996). – Japan.

   Pullulan, an extra cellular polysaccharide excreted by the fungus *Aureobasidium pullulans*, is generally recognized as safe (GRAS), through scientific procedures, for use in food in general, for multiple technical effects. –US

   Identified substance in cosmetics and personal care products regulated under the Food and Drugs Act that was in commerce between January 1, 1887 and September 13, 2001. -Canada

2. Manufacturer: Hayashibara Co. LTD.
   2-3, Shimoishii 1-chome
   Okayama, 700 Japan

   Phone: (086)224-4311
   FAX: (086)225-5630
   (086)221-0534

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3. Pullulan (dry basis) is used as a film forming agent in the manufacturing process of empty polysaccharide capsules, for the purpose of encapsulating dietary supplement ingredients. The level of pullulan (dry basis) in the pullulan film as used in the capsule application identified herein is approximately 80% or greater, with water being the next highest constituent.

4. See Illustration 1

5. Pullulan (dry basis) is a natural, water-soluble, polysaccharide consisting of glucose (glucan) with a simple linear structure wherein maltotriose units (three glucose molecules joined through alpha – 1, 4-glucosidic bonds) are repeatedly polymerized through alpha - 1, 6-glucosidic bonds.

Manufacturing Process:

Pullulan (dry basis) is produced through fermentation of black yeast, Aureobasiduium pullulans, in a starch syrup culture at 22° - 30°C. Strains of A. pullulans are used which produce the lowest amount of black-pigment and which require the shortest cultivation periods to yield maximum quantities of pullulan. Pullulan is elaborated extra cellular into the culture medium from which it is recovered and purified.

Culture	
|	
Cultivation	Seed Culture/Air
|	
Sterilization	(Filtration or centrifugal separation)
|	
Purification	(Decolonization) membrane treatment ion exchange
|	
Desalination	
|	
Concentration	
|	
Drying	Drum drying, spray drying
|	
Pulverization	

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Pullulan (dry basis)

The strain of microorganisms used for the production of pullulan is the "Hayashibara strain." At the time of cultivation, stock cells are taken from ampules and streaked on agar plates. If, after colony formation, the purity of the culture is confirmed, one colony is transplanted to an agar slant. The transplanted colony is then used as the inoculum for the production of pullulan.

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

Live organisms of the Hayashibara strain are killed by heat sterilization in the course of producing pullulan. The absence of the live strain in product is determined by dissolving Pullulan in sterilized water which is added to an agar plate able to support growth of *A. pullulans*.

6. See attachment A (Agency Response Letter GRAS Notice No. GRN 000099)

7. See attachment B (Opinion of the GRAS Expert Panel on the Safety of Pullulan as a Food Ingredient)

8. CAS Number – 9057-02-7

Product that contains Pullulan - Fresh Burst™ Listerine PocketPaks™, Oral Care Strips, Product number 07-0354-29; Dist: Pfizer Consumer Healthcare, Morris Plains, NJ 07950 USA

9. See attachment C (Physical properties and etc.)

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10.

See attachment D (Pullulan Safety Data)

See attachment E (Safety studies of a novel starch, pullulan)

See attachment F (Material Safety Data Sheet)

Pullulan

Pullulan is a modified starch produced by naturally occurring yeast, *Aureobasidium pullulans*. The basic unit of pullulan consists of a series of three alpha-1, 4 linked glucose molecules repeatedly polymerized *via* alpha-1, 6 linkages on the terminal glucose. Moreover, pullulan and the associated yeast have a substantial history of use in foods in Japan, and pullulan is structurally and metabolically similar to starch.

Metabolism Studies

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. The majority of the administered pullulan is fermented in the large intestine to short-chain fatty acids (SCFAs). Thus, the digestion and metabolic fate of pullulan is similar to that of normal starches.

Human Safety Data

Pullulan has been used in Japan, in various forms, for more than 20 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.

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Nonclinical Safety Data

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 LD_{50} 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 to 40%) can be attributed to the effect of replacing the normal nutrient content of food with indigestible carbohydrate (LRSO, 1975).

The NO (A) EL 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 cecal 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).

Genotoxicity/Mutagenicity Data

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).

Conclusion

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 20 years of

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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.

Although the above profile provides a general overview of the safety data for pullulan, the use of products containing this compound should be assessed within the context of anticipated exposure levels.

11. Review and Research

References

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Ishidate, M.; Sobuni, T.; Kishi, M. 1985. Mutagenicity study results: Natural food additives (Part 6). Tokishikoroji Foramu Toxicol Forum 8(6):705-708.

Kimoto, T.; Shibuya, T.; Shiobara, S. 1997. Safety studies of a novel starch, pullulan: Chronic toxicity in rats and bacterial mutagenicity. Food Chem Toxicol 35(3):323-329.

Kuroda, K.; Yoo, Y.S.; Yoshikura, T.; Ishibashi, T. 1985. Mutagenicity of natural food additives. Annu Rep Osaka City Instit Public Health Environ Sci 47:24-30.

LSRO. 1975. Evaluation of the Health Aspects of Dextrin and Corn Dextrin as Food Ingredients. Prepared by Federation of American Societies for Experimental Biology (FASEB), Life Sciences Research Office (LSRO), Bethesda, Maryland for Food and Drug Administration, U.S. (FDA), Bureau of Food, Washington, DC.

MacKenzie, K.M.; Hauck, W.N.; Wheeler, A.G.; Roe, F.J.C. 1986. Three-generation reproduction study of rats ingesting up to 10% sorbitol in the diet: And a brief review of the toxicological status of sorbitol. Food Chem Toxicol 24(3):191-200.

Okada, K. Yoneyama, M.' Mandai, T.; Aga, H.; Sakai, S.; Ichikawa, T. 1990. Digestion and fermentation of pullulan. Eiyo to Shokuryo 43(1):23-29 (Includes Translation).

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Oku, T.; Yamada, K.; Hosoya, N. 1979. Effect of pullulan and cellulose on the gastrointestinal tract of rats. Eiyo to Shokuryo 32(4):235-241 (Includes Translation).

Olivier, P.; Verwaerde, F.; Hedges, A.R. 1991. Subchronic toxicity of orally administered beta-cyclodextrin in rats. J Am Coll Toxicol 10(4):407-419.

Yoneyama, M.; Okada, K.; Mandai, T.; Aga, H.; Sakai, S.; Ichikawa, T. 1990. Effects of pullulan intake in humans. Denpun Kagaku (J Jpn Soc Starch Sci) 37(3):123-127 (Includes Translation).

[2] Listed for use in marketed cosmetic and personal care products.

12.

Pullulan

What are pullulan capsules?

- Capsules composed of an edible material derived from corn syrup

What is this material?

- Its formal chemical name is pullulan (not a brand name...just like "gelatin" is not a brand)
- It is a carbohydrate, and is considered a "simple polysaccharide"
- Textbook definition of pullulan:
  - "A water-soluble polysaccharide composed of glucose units that are polymerized in a way as to make it viscous and impermeable to oxygen."

What does pullulan look like?

- Tasteless, odorless white powder
- Solutions of pullulan can dry to films
- Films are quickly soluble in water, have low oxygen permeability and are glossy

How long has it been around?

- First reported in 1938

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- First commercial production in 1976, as a food additive (e.g. thickener); consumed safely in Japan for over 20 years
- Most recognized US use of pullulan is the film for Listerine Pocketpaks™

How is pullulan made?

- Uses a fermentation process, where a yeast-like organism (*aureobasidium pullulan*) acts on a food source (*corn syrup; certified GMO-free*)
- From Corn to Pullulan:
  - Corn starch comes from "starchy" (chains of glucose) part of corn. Starch is how plants store energy (glucose)
  - To make corn syrup, enzymes are added to corn starch, to turn it into a syrupy mixture of simple sugars (e.g. glucose, dextrose, maltose)
  - *A. pullulans* acts on corn syrup, stringing the sugars together into a simple polysaccharide called pullulan
  - In your body, pullulan is digested like many other common carbohydrates

Pullulan Production Overview

- Conducted under GMP conditions; raw materials meet food grade specifications
- Fermentation of corn syrup with *A. pullulans*
- Micro-filtration to remove *A. pullulans*
- Cell-free filtrate is heat sterilized; absence of *A. pullulans* is confirmed
- Filtrate is decolorized, filtered and de-ionized; then evaporated; then filtered again
- Dried and pulverized to form granules

More About A. Pullulans

- Abundant in nature (decaying trees, exterior windows, breweries, paper mills, etc.)
- Non-toxic

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- Non-pathogenic
- Human consumption study showed no symptoms; clear evidence that colonic activity completely digests pullulan
- Long-term rat studies show no negative effects

Regulatory Status

- US
  - GRAS (Generally Recognized as Safe)
    - Granted on Aug. 1, 2002 by FDA
    - GRAS Notice #: GRN 0000999
- Japan
  - Approved as food ingredient and additive; no limitation of use
    - Serial #373; April 16, 1996
- Europe
  - Under review as Food Additive

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Petition For Listing Of Pullan On The USDA National List Of Allowed And Prohibited Substances

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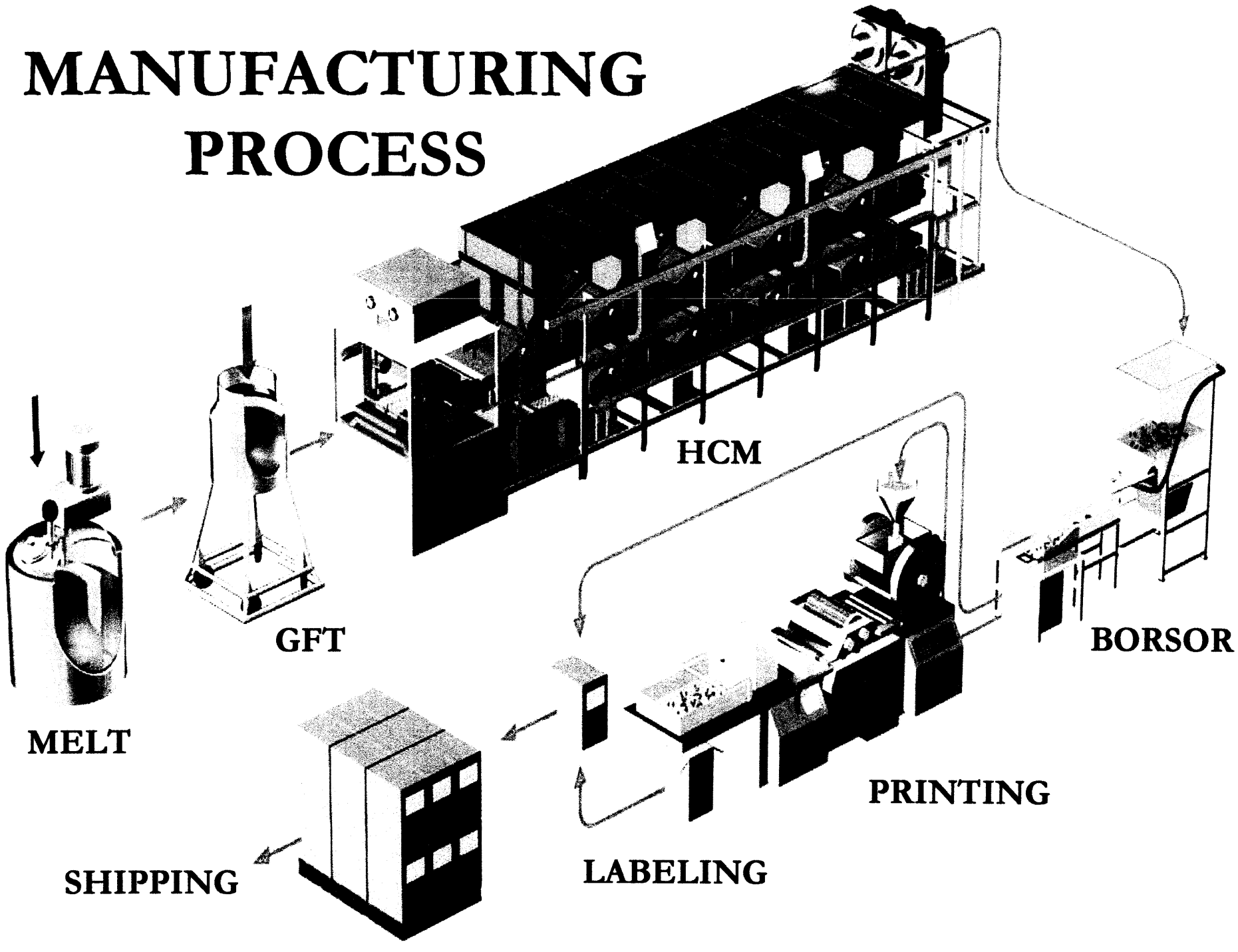
MANUFACTURING PROCESS



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-glucosidic 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 a 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 Hyashibara 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 Hyashibara 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.

OPINION OF THE GRAS EXPERT PANEL ON THE SAFETY OF PULLULAN AS A FOOD INGREDIENT

The Expert Panel was requested by the Warner-Lambert Company to determine the GRAS status of pullulan for use as a flavor substrate. The Expert Panel independently reviewed the material submitted by the Warner-Lambert Company and other relevant information. The Expert Panel then met with representatives of the Warner-Lambert Company during which additional information was provided. The expertise of the Expert Panel is documented by the attached C.V.'s.

Pullulan is a natural polysaccharide elaborated extracellularly by Aureobasidium pullulans. It is commercially produced by a non-pathogenic and non-toxic strain of A. pullulans utilizing starch and other food grade components. Pullulan has a linear structure comprised of maltotrioses in which three glucose units are linked through alpha 1,4-glucosidic bonds. The maltotrioses are in turn linked to a series of other maltotrioses through alpha 1,6-glucosidic bonds creating a long stair-step type structure. The molecular weight range for pullulan is 50,000-500,000 daltons.

Pullulan is virtually identical in composition to dextrin and maltodextrin, which have been affirmed GRAS under 21 CFR Part 184, in the sense that all three substances consist of glucose units linked through alpha 1,4-glucosidic bonds as well as through alpha 1,6-glucosidic bonds. Maltodextrin, which was recently affirmed GRAS by FDA, consists of approximately 20% alpha 1,6-glucosidic bonds while pullulan contains approximately 30% alpha 1,6-glucosidic bonds. All three of the above substances are similar to food starches that are composed of amylose and amylopectin fractions. A typical food starch, such as corn starch, consists of 95% alpha 1,4-glucosidic bonds and 5% alpha 1,6-glucosidic bonds.

Commercial pullulan is produced with a purity and quality comparable to that specified for dextrin (Food Chemicals Codex, 3rd Edition).

Pullulan is approved as a food ingredient in Japan by the Food Chemical Section, Environmental Health Department, Ministry of Health and Welfare. 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.

Pullulan film has been used in Japan for more than 20 years as both an indirect food ingredient for coatings on food packaging and directly in food for a variety of applications. These applications include use for food decoration as a mixture with other ingredients such as flavors or coloring agents to decorate various foods popular in Japan. Pullulan film can also be made by cutting or slicing the film to desired shapes or pieces called chips that are used as decoration for candy, cake and other bakery items. The film is also cut into sheets and used as a binder for seasoning that is intended for wrapping various food items. Another application is the use of pullulan film as edible packaging material.

The intended use of pullulan, which is the subject of this Expert Panel's review, is as a film, containing flavoring agents and artificial sweeteners, which would be consumed as a mouth freshener. Mouth fresheners consisting of pullulan film with added flavors are consumed in Japan. However, this would be a new product for the U.S. and the nearest related food category is probably breath mints.

Using the consumption patterns of breath mints in the U.S. as a basis for exposure assessment, the estimated intake of pullulan is considered to be less than 4 mg/kg b.w./day.

Studies conducted on the digestion of pullulan have demonstrated that only partial hydrolysis occurs by salivary and pancreatic amylases without glucose formation. Slight amounts of glucose are generated by small intestinal enzymes, but the majority of pullulan is fermented in the large intestine to short chain fatty acids similar to starches, dextrin and maltodextrin. The only difference in the digestion of these substances is attributable to the differing percentages of alpha 1,6-glucosidic bonds. As pullulan contains a higher percentage of such bonds, less of it is digested in the upper gastrointestinal tract and consequently more is digested in the lower bowel. Typical diets normally contain several grams of alpha 1,6-linked glucose units compared to only milligram quantities of alpha 1,6-linked glucose units from the intended use of pullulan film.

The safety of pullulan can be determined based on the chemical nature and composition of pullulan, the means by which it is manufactured and the chemical purity of the final product. Since pullulan is chemically identical to other GRAS substances such as food starch and dextrin, it is considered inherently safe under its intended conditions of use. Any safety concern focuses on the possible presence of impurities. As the substances used in the fermentation process are all food-grade and given that the strain of A. pullulans employed in the fermentation process is non-pathogenic and non-toxic, there is no safety concern over the presence of contaminants. Further, chemical specifications for pullulan will be comparable to those established for dextrin and maltodextrin in the FCC.

The safety of the use of pullulan film as a flavor substrate is further supported by 20 years of use of pullulan as an ingredient of human food. Additionally, a short term, high-dose study in humans was conducted in 1990 in which no adverse or untoward effects from pullulan consumption were observed.

A study corroborating the safety of pullulan was conducted in Sprague-Dawley rats in which pullulan was administered as a dietary admixture at levels of 1, 5, and 10% for a period of 62 weeks provided a NOAEL equal to or greater than 4,450 mg/kg b.w./ day in males and 5,080 mg/kg b.w./day in females. The only treatment related change noted was an increase in cecal weights in female rats receiving the highest concentration (5,080 mg/kg b.w./day) of pullulan in their diets. These findings support an acceptable daily intake of at least 45 mg/kg b.w. day as an ingredient in food. The mutagenicity of pullulan was assessed with and without metabolic activation in Salmonella typhimurium (TA 98, 100, 1535 and 1537) and determined to be non-mutagenic in this system.

Based on its review and deliberations and assuming pullulan is produced under current good manufacturing practice and is of food grade purity, the Expert Panel concluded that the intended use of pullulan film as a direct ingredient for mouth freshener is safe and generally recognized as safe by established scientific procedures.

EXPERT PANEL

Joseph F. Borzelleca, Ph.D. Date 17 April 1996

George A. Burdock, Ph.D. Date 17 April 1996

W. Gary Flamm, Ph.D. Date 17 April 1996

C.V.'s attached (3)

e. Quantitative composition.

Pullulan is a natural, water-soluble, polysaccharide consisting of glucose (glucan) with a simple linear structure wherein maltotriose units (three glucose molecules joined through *alpha*--1,4-glucosidic bonds) which are repeatedly polymerized through *alpha*-1,6-glucosidic bonds. Fine structural studies (Catley, 1971) revealed a few percent of maltotetraose substituting for maltotrioses.

f. Method of manufacture.

Pullulan is produced through fermentation of black yeast, *Aureobasidium pullulans*, in a starch syrup culture at 22°- 30°C. Strains of *A. pullulans* are used which produce the lowest amount of black-pigment and which require the shortest cultivation periods to yield maximum quantities of pullulan. Pullulan is elaborated extracellularly into the culture medium from which it is recovered and purified as described in the following narrative.

Method of Manufacture - Pullulan Polysaccharide		
Sequence number and operation	Narrative	Materials and additives*
1 - Preparation of material.	The main propagation culture medium is prepared by adding a small quantity of inorganic nitrogen salt to the partially hydrolyzed starch suspension.	Corn syrup, sodium chloride, Brewer's yeast extract, magnesium sulfate, dipotassium phosphate, sodium glutamate, ammonium sulfate, diammonium phosphate and silicon oil (KM72).
2 - Sterilization of culture medium.	The main propagation culture medium is sterilized.	
3 - Propagation of seed culture.	Prepare *Aureobasidium pullulans* seed culture in culture medium of the same composition as for the main propagation.	Seed culture.
4 - Main propagation.	Inoculate seed culture into culture medium and effect main propagation under aeration by stirring for 3 to 7 days until extracellular production of pullulan reaches a maximum.	Hydrochloric acid.
5 - Neutralization.	Neutralize the culture mixture if necessary.	Calcium hydroxide.
6 - Removal of cells.	Remove cells with a precoated filter.	Diatomaceous earth.

7 - Purification	Decolorize and deionize the pullulan solution	Hydrochloric acid, activated carbon, anion exchange resin, cation exchange resin and sodium hydroxide.
8 - Condensation.	Condense the pullulan solution 20-40% on a dry solid basis.	
9 - Drying.	Dry the solution with a drum dryer.	
10 - Pulverization	Pulverize the resultant pullulan mixture into the desired particle sizes to obtain tasteless and odorless pullulan as a fine white powder.	
11 - Final product		
All materials used are foods or ingredients are cited for the purposes employed in 21 CFR.		

Pullulan film is prepared by dissolving pullulan powder in water to give a 15-20% pullulan solution. The solution is coated on a substrate *e.g.*, plastic film, and then dried at 70-80°C for form a film.

g. *Aureobasidium pullulans*.

The taxonomy of *Aureobasidium pullulans* has been described in the literature (Cooke, 1961). The strain of microorganism used for the production of pullulan is the "Hayashibara strain." *Aureobasidium pullulans* is generally regarded as being non-pathogenic and non-toxigenic to mammals (discussed in Kimoto *et al* 1997 and Wallenfels *et al* 1961).

h. Specifications for food grade material.

Parameter	Specification
Appearance	White powder
Pullulan (dry basis)	>90%
Oligo-saccharides (dry basis)	<10%
Moisture	<6.0%
Ash	<1.5%
Viscosity	100-180 cst (10 wt.%, 30°C)
Lead	<1 ppm
Arsenic	<2 ppm
Heavy metals	<5 ppm
pH	5.0-7.0
Coliforms	<10/g maximum
Yeast and molds	<100/g maximum
Salmonella	0/25 g
E. coli	0/25 g
Staphylococcus aureus	0/25 g

3. **Information on any self-limiting levels of use.**

Pullulan as a flavor carrier for mouth fresheners would share the same self-limiting qualities as other currently-marketed mouth fresheners (*e.g.* Certs Cool Mints and Certs Extra Flavor).

4. **The determination that pullulan polysaccharide is GRAS is on the basis of scientific procedures.**

See attached - OPINION OF THE GRAS EXPERT PANEL ON THE SAFETY OF PULLULAN AS A FOOD INGREDIENT

5. **Selected references**

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Pullulan

Pullulan is a modified starch produced by a naturally occurring yeast, *Aureobasidium pullulans*. The basic unit of pullulan consists of a series of three alpha-1,4 linked glucose molecules repeatedly polymerized *via* alpha-1,6 linkages on the terminal glucose. Moreover, pullulan and the associated yeast have a substantial history of use in foods in Japan, and pullulan is structurally and metabolically similar to starch.

Metabolism Studies

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. The majority of the administered pullulan is fermented in the large intestine to short-chain fatty acids (SCFAs). Thus, the digestion and metabolic fate of pullulan is similar to that of normal starches.

Human Safety Data

Pullulan has been used in Japan, in various forms, for more than 20 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.

Nonclinical Safety Data

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 LD_{50} values of >24 and >40 g/kg body weight in male dd 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 to 40%) can be attributed to the effect of replacing the normal nutrient content of food with indigestible carbohydrate (LRSO, 1975).

The NO(A)EL 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 cecal weight in female rats receiving 5,080 mg/kg body weight/day of pullulan (about 10% of diet). Increased cecal 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).

Genotoxicity/Mutagenicity Data

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).

Conclusion

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 20 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. Although the above profile provides a general overview of the safety data for pullulan, the use of products containing this compound should be assessed within the context of anticipated exposure levels.

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JOSEPH F. BORZELLECA, PH.D.
PROFESSOR, E. S.

RE: Safety studies of a novel starch, pullulan: chronic toxicity in rats and bacterial mutagenicity
MS 423/96

Dear Gary:

Thank you for your willingness to respond to the comments of the referees regarding your manuscript. I am please to inform you that your article has been accepted for publication in *Food and Chemical Toxicology*.

The manuscript will be forwarded to Stella Hill, the Assistant Editor of Food and Chemical Toxicology, where the manuscript will be copyedited, sent to the printer for typesetting, and published in the next available issue. Please return your galleys promptly and limit your changes to minor editoral or typesetting corrections.

Thank you for submitting your study to *Food and Chemical Toxicology* and we trust you will continue to support the aims of FCT by submitting the results of your investigations.

Cordially,

J. F. Borzelleca/sa H

Joseph F. Borzelleca
Professor, Pharmacology and Toxicology
Editor, *Food and Chemical Toxicology*

Reference MS423/96

SAFETY STUDIES OF A NOVEL STARCH, PULLULAN: CHRONIC TOXICITY IN RATS AND BACTERIAL MUTAGENICITY*

by

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*The rat study was conducted at the School of Medicine, Juntendo University, Tokyo, Japan. The mutagenicity study was conducted at Hatano Research Institute, Food and Drug Safety Center.

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ABSTRACT

This study was designed to assess the potential toxicity of pullulan, a starch like substance produced by Aureobasidium pullulans proposed for use as a texturizer for meat and meat substitutes and as a flavor substrate. Sprague-Dawley rats (15/sex/group) were administered pullulan as a dietary admixture at levels of 1, 5 and 10% for a period of 62 weeks. Control animals (15/sex) received untreated standard laboratory diet. The feeding study, originally intended to continue for 24 months, was terminated at 62 weeks due to poor survival resulting from intercurrent pneumonia which was confirmed by histological findings. At 62 weeks of treatment, all survivors were sacrificed, complete gross postmortem examinations were conducted on all animals, selected organs were weighed and organ/body weight and organ/brain weight ratios calculated. Mean body weights of all treated groups were comparable to controls. There were no indications of an adverse effect of pullulan on food consumption or food efficiency. At termination of the study, hematology and clinical chemistry values of treated animals were comparable to control values. There was no indication of pullulan related toxicity in terminal organ and body weights. Macroscopic and microscopic examinations revealed no toxic lesions due to the treatment. The mutagenicity of pullulan was assessed both with and without metabolic activation in Salmonella typhimurium (TA 100, 1535, 98, and 1537). 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 suggesting it lacks mutagenic/carcinogenic potential. Based on these results, it is concluded that pullulan lacks significant toxicologic activity. The NOAEL was 10% (equal to or greater than 4450 mg/kg bw/day in males and 5080 mg/kg bw/day in females) which would support use in various foods as a substrate for flavors.

INTRODUCTION

Pullulan is a water-soluble viscous polysaccharide, an alpha-glucan, consisting of glucose units with a relatively simple linear structure, i.e., units of three alpha-1,4 linked glucose molecules that are repeatedly polymerized *via* alpha-1,6 linkages on the terminal glucose (Walenfels et al., 1965). Typical food starches, such as corn starch, consist of 20% amylose (alpha-1,4 linked glucose molecules) and 80% amylopectin which contain both alpha-1,4 and alpha-1,6 glucose linkages (Fruton and Simmonds, 1959). For pullulan, however, the alpha-1,6 linkage serves to cross-link individual short chains resulting in a stair-step structure (Figure 1). As pullulan has a weight average molecular weight of 50,000-500,0000, n in Figure 1 ranges from 300 to 3,000.

Pullulan is elaborated extracellularly by "black yeast", *Aureobasidium pullulans*. The organism is generally regarded to be non-pathogenic and non-toxigenic (Wallenfels et al., 1961) although there are reports of its presence in clinical samples of blood and tissue (Salkin *et al*. 1986; Kaczmarski *et al*. 1986) and possible pathogenicity in an immunocompromised woman associated with a chronic indwelling catheter (Girardi *et al*. 1993). However, as pointed out by Ajello (1978), the mere isolation of a fungus from a lesion or from clinical material does not *per se* establish the isolate as a pathogen. Similarly, its growth in an immunosuppressed individual with a chronic indwelling catheter may be more a saprogenic than pathogenic characteristic. In this

connection, it should be recognized that Saccharomyces cerevisiae (brewer's or baker's yeast), generally regarded as completely harmless, has been implicated in vaginitis in predisposed patients (Sobel et al. 1993), invasive fungal infections in immunosuppressed transplant recipients (Tollemar et al. 1992), and pneumonia in a patient with AIDS (Tawfik, 1989). There is also a report on the isolation of three new mycotoxins produced by Aureobasidium pullulans (Schrattenholz and Flesch, 1993). These substances have not been shown to be toxic to mammals nor are they similar in structure to known classes of mycotoxins. As the strain of Aureobasidium pullulans used here was orally administered to dd strain of male mice and male and female Sprague-Dawely rats as an aqueous suspension of a lysate of the organism at a dose of 24 g/kg and 40 g/kg, respectively, without resulting in mortality (reports submitted by School of Medicine, Juntendo Univeristy, Tokyo, Japan and Mitsubishi Chemical Safety Institute, for the mouse and rat studies, respectively). It is concluded that the subject strain is non-toxic. The organism is commonly found in breweries and distilleries. For commercial production, strains of Aureobasidium pullulans with the lowest black pigment production are selected and then further mutated and selected to enhance yields of pullulan.

Because of pullulan's unique properties and characteristics (i.e. extremely high water solubility, film forming capacity, and gum like properties), it has many potential food uses including use as a component of flour, as a texturizer for "tofu", ham and

sausage, and as a substrate for flavors and as a means of protecting flavors through microencapsulation. Coatings containing pullulan have been proposed as a substitute for paraffin to preserve freshness of eggs. Pullulan film has been used for various food applications in Japan. Such applications include: Food decorations for candies and bakery goods and in beverages; as a binder for seasoning and a sheet for wrapping various food items; and as edible packaging material for instant noodles or packages of table top sweeteners. Commercial interest in the U.S. is currently focused on the application of pullulan film as a flavor substrate for a mouth-refreshing film (mint-like) item which is commercially available in Japan. Exposure levels from this use are estimated to be less than 4 mg/kg bw/day.

Studies by Okada et al., (1990) on the digestion and fermentation of pullulan have demonstrated that only partial hydrolysis occurs by salivary and pancreatic amylase without glucose formation while slight amounts of glucose are formed by small intestinal enzymes. Mostly, pullulan is fermented to short-chain fatty acids in the large intestine similar to other slowly digestible carbohydrates (Okada et al., 1990).

Pullulan is expected to be non-toxic based on its dextran and starch-like composition and structure (Clevenger et al., 1988), and its high degree of purity. Pullulan was administered to healthy human volunteers at a level of 10 g pullulan/day for 14 days

(Yoneyama et al., 1990). No adverse effects were reported. Chronic feeding studies in rats and mutagenicity studies using Salmonella typhimurium tester strains, TA 100, TA 1535, TA 98 and TA 1537 were conducted to confirm the safety of pullulan. Results of these studies can be used to assess the safety of pullulan when used as an ingredient in food.

MATERIALS AND METHODS

1. Test Substance

Pullulan was produced by cultivation of Aureobasidium pullulans on a medium supplemented with effective carbon and nitrogen sources and minerals under aeration. Pullulan was recovered from the culture fluid by centrifugation to remove cultured cells, fractionated with alcohol and purified. Specifications for pullulan have been established by Hayashibara for use as a food ingredient in Japan (Sugimoto, 1978) as: residue on ignition, not more than 4.0%; pH, 5.0-7.0; viscosity at 30 °C and 100 w/w%, 100-180 cst; protein, not more than 0.3%; heavy metals as lead, not more than 5 ppm; arsenic as As_2O_3, not more than 2 ppm, viable colony count, not more than 300 colonies/g.

2. Test Animals

Sprague-Dawley rats, JCL strain, were supplied by Japan Clea Company, Ltd. The animals (120 rats equally divided among males and females) were 4 weeks old at receipt. They were divided into 4-dose groups, including untreated controls, consisting of 15 animals per sex per dose group (Table 1). They were housed 5 rats of either sex per cage equipped with an automatic water-supplier and maintained at a temperature of 23± 1°C and a relative humidity of 55± 5%. At the initiation of treatment, the mean body weight (X ± S.D.) for individual groups were: 61 ± 3 g (10%), 62 ± 3 g (5%), 61 ± 4 g (1%), 62 ± 4 g (controls), males; 64 ± 2 g (10%),

64 ± 3 g (5%), 64 ± 3 (1%), 64 ± 3 g, females. Food and water were provided ad libitum. The animals were observed once daily for mortality and gross signs of toxicity or pathology. Food consumption and body weights were determined weekly.

3. Selection of Dose

Pullulan was determined to be non-toxic when orally administered as a single dose to male dd strain mice (LD_{50} > 14.3 g/kg, suspended in olive oil), the highest amount technically possible to administer. In consideration of this finding, pullulan was administered in the diet at a highest level of 10% added directly to a diet. Higher levels of pullulan were not considered because of the possibility of confounding effects resulting from nutritional imbalances. An intermediate concentration of 5% was chosen consistent with the U.S. National Toxicology Program dose selection procedures and a low dose, one-tenth of the high-dose selected or 1%, consistent with FDA guidelines for toxicological testing of food additives (FDA,1982). The diet (CE-2, Japan Clea Co. Ltd., Tokyo, Japan) consisted of: crude protein, 24%; crude fat, 3.5%; crude fiber 4.5%; carbohydrate, 56%; ash, 6%; moisture, 6.0%. Analysis of the mineral content of the diet revealed: calcium, 1%; phosphorous, 1%; magnesium, 0.27%; sodium, 0.31%; potassium, 0.85%; manganese, 60 ppm; and iron, 100 ppm.

4. Hematology and Serum Biochemistry

At termination of the study, rats were lightly anesthetized with ether and blood was collected directly from the heart. For hematology, the following was determined: RBC and WBC

(Automatic hemocytometer, Coulter Counter D-type, Coulter Electronics), hematocrit and hemoglobin (Cyan-methemoglobin method, Hitachi Spectrophotometer 101) and differential count. The following clinical chemistry determinations were made: serum transaminase (ALT, AST) (Reitman Frankel method, Hitachi: Spectrophotometer 101); alkaline phosphatase (SAP) (Auto Analyzer Technician A-2); cholinesterase (ChE) (Mickel Ueda method); albumin globulin ratio (A/G) (Bromocresol green method. Auto Analyzer Technicon A-2); total cholesterol (Cho) (Orthophthalaldehyde method, Vickers D-300, Vickers Ltd.); blood sugar (Neocuproin method, Auto Analyzer Technicon A-2, Technicon Instruments Corp.)

5. Urinalysis

Fresh urine, collected at necropsy, was examined for protein, sugar, ketone bodies, pH and occult blood (Ames, Labstick, Miles, Sankyo Co., Ltd., Tokyo, Japan).

6. Necropsy, Measurement of Organ Weight, and Histopathology

After taking blood samples for analysis, animals were sacrificed by exsanguination and the following organs were examined macroscopically and microscopically (tissues fixed in 10% formalin and stained with hematoxylin and eosin) in all animals: brain, heart, lungs, liver, kidneys, spleen, adrenals, stomach, small intestine, rectum, ovaries, uterus, testes, trachea, urinary bladder, submandibular glands, thyroid and sternum (bone marrow). The following organs were weighed: brain, heart, lungs, liver, spleen, kidneys, stomach, testes, uterus, ovaries, adrenals, submandibular glands.

7. Gene Mutational Assay Using Bacteria

Salmonella typhimurium strains TA 100, TA 1535, TA 98 and TA 1537 were obtained from Dr. Bruce Ames at the University of California. The plate incorporation method as described by Ames et al., (1975) was followed. This involved mixing 0.1 ml of bacterial suspension with a 0.1 ml of test solution containing various concentrations of pullulan and 0.5 ml of S-9 mix (Ames et al., 1975) or phosphate buffer and added to 2 ml of molten soft agar (containing 0.05 mM biotin and 0.05 mM histidine) which was poured onto a minimal-glucose agar plate (0.2 g $MgSO_4$ $7H_2O$, 2 g citric acid, 10 g K_2HPO_4, 3.5 g Na (NH_4) HPO_4 $4H_2O$, 20 g glucose 15 g agar and 1 liter distilled water) and incubated at 37 °C for 48 hours prior to counting the number of mutant colonies.

The S-9 mix consisted of S-9, supernatant of a 9,000 g induced rat liver homogenate (500 mg/kg PCB induced), 8 mM $mgCl_2$, 33 mMKCl, 5.8 mM G6P, 4.6 mM NADPH, 100 mM sodium phosphate buffer at pH 7.4.

RESULTS

The duration of the study was initially intended to be 24 months but because survival of the male control, mid- and high-dose groups fell below 50%, the study was terminated after 62 weeks. Survival among female controls was good, 86.7%, but poor, 40%, among the female high-dose group. Examination at necropsy indicated deaths were due to pneumonia and were not regarded as treatment related by the pathologist. Survival curves for males and females are given in Figures 2 and 3, respectively.

No dose-related, statistically significant differences were observed in body weight. Mean body weights in males and females of all dose groups increased rapidly from initiation of the study until about the tenth week. Males continued to gain weight gradually until they reached about 600 g, while females grew more slowly achieving mean weights of about 350 g by the end of the study (week 62). Final mean body weights are presented in Table 2.

Food consumption values in the treated groups were comparable to the values of the control group. No increase in mean food consumption values occurred after the second week in either males (about 23 g/day) or females (about 15 g/day). As both males and females gained weight rapidly from the beginning of the study until week 10, the mean daily consumption per kilogram of body weight declined sharply during this period. Intake of pullulan in males

of the 10% group decreased from 12 g/kg bw/day at week one to 5.9 g/kg bw/day by week 10 (5% group declined from 7.2 to 2.9 g/kg bw/day; 1% group declined from 1.3 to 0.58 g/kg bw/day). In females fed 10% pullulan the decrease for the same period was from 11 to 5.5 g/kg bw/day (5% group declined from 6.3 to 3.3 g/kg bw/day; 1% group declined from 1.2 to 0.62 g/kg bw/day). No dose related differences in final body weight were noted in either males or females.

Values for hematological and clinical chemistry parameters for all treated groups at study termination were comparable to controls with only a few exceptions. None of the exceptions suggested a treatment or dose-related effect. Among males, the RBC count in the 10% dose group was elevated compared to the control value (684 $\pm$ 147 x $10^4/mm^3$ versus 506 $\pm$ 94 x $10^4/mm^3$, $p< 0.05$). However, the 1% group exhibited an even higher value, 694 $\pm$ 69 x $10^4/mm^3$, while the value for the mid-dose group (5%) was virtually identical to the control's, 514 $\pm$ 154 x $10^4/mm^3$. Similarly, a statistically significant decrease in the percent lymphocytes in mid- and low-dose males, 61 $\pm$ 16% and 66 $\pm$ 9% versus 81 $\pm$ 8% for controls, was not dose-related as the high-dose group was virtually identical to controls with a value of 78 $\pm$ 9%. There were no cases, other than the one identified above, in which high-dose groups of either males or female animals showed statistically significant increases compared to their corresponding control for the parameters measured i.e., hemoglobin concentration; hematocrit, RBC count (exception

noted); white blood cell count; mature leukocyte count (lymphocytes, monocytes, neutrophils, eosinophils, basophiles); blood sugar concentration; total cholesterol; total protein; A/G ratio; ALT; AST; SAP; serum cholinesterase.

Absolute organ weights, in both males and females (Tables 3 and 4), varied from controls but in a non-treatment related manner and no statistically significant differences occurred when compared as relative weights (organ weight/body weight).

Mean cecum weight for females of the high-dose group was about 40% higher than controls, statistically significant with a $P<0.01$. No significant effect on mean cecum weights was observed for male groups. The increase in mean cecum weight for the female is considered a physiological response to pullulan which is not digested completely in the upper gastrointestinal tract.

Postmortem macroscopic examination of tissues in male animals revealed principally pneumonia and pulmonary abscesses. In females, less pneumonia and fewer pulmonary abscesses were found than in males but other lesions were noted: ovarian cyst, cerebral hematoma and uterine hematoma in the mid-dose group; myoma, subcutaneous abscess, uterine hematoma in the low dose; myomas, hepto-abscesses, spleno-abscesses, subcutaneous abscesses and uterine cyst in controls. Histological examination of macroscopic lesions agreed with macroscopic observations. Bronchitis was

observed histologically in all male and female groups. In the absence of dose-related effects no histopathology was attributed to treatment with pullulan. The only histopathological finding was that common to Sprague-Dawley rats between one and two years of age: i.e., myocarditis (one in each of the following male groups: 5%, 1%, and control), nephritis (one each in 5% and 1% male groups), tracheal calcification (one in 10% female group), splenic hemosiderosis (one each in 10% and 5% female groups), uterine squamous cell metaplasia (one in 10%), and spermatogonial hypoplasia (one in 5% group).

No significant increase in mutant colonies per plate was observed for any tester strain either with or without metabolic activation with S-9 mix at concentrations of pullulan up to 10,000 μg per plate (Table 5). The standard positive controls used in the spot tests elicited the expected mutagenic responses indicating the tests were valid (Table 5).

DISCUSSION

Pullulan, administered in the diet for 62 weeks at concentrations up to 10%, produced no treatment related adverse effects in male or female Sprague-Dawley, JCL, rats. Poor survival due to pneumonia led to termination of the study after 62 weeks. While female controls showed better survival (87%) than the treated groups (1%, 67%; 5%, 67%; 10%, 40%), no dose dependency was noted between pullulan intake and survival among males. Survival in the 10% pullulan group (47%) was comparable to controls (47%), the 1% pullulan group showed slightly better survival (60%) while the 5% pullulan group survival (27%) was less than controls. Hematological and clinical chemistry parameters of all treatment groups were comparable to controls with the exception of a few spurious differences which were not dose- or treatment- related.

The terminal body weights in male and female treatment groups were regarded as comparable to control groups. Although, the low-dose and high-dose male groups had terminal mean weights that were significantly lower than controls ($p < 0.05$), there were no dose-related trends, therefore, the findings are considered unrelated to treatment. Food consumption and food-efficiency (body weight gain/weight of food consumed) were comparable for all groups.

While the absolute mean organ weights among treated male groups were slightly but significantly higher (brain) or lower

(liver, kidney, submandibular gland) in certain cases than the corresponding mean for controls, the ratio of organ weight to body weight of treated male groups was not significantly different from controls. In contrast, mean organ weights for certain female treatment groups were slightly but significantly higher (heart, liver, spleen) or lower (brain) than controls but no statistically significant differences were found for organ to body weight ratios. The absence of any statistically significant difference in organ to body weight ratios between control and treated groups indicates the effects are not treatment-related. Additionally, no histological changes were observed to indicate that these organ weight differences were meaningful.

The only change in organ weight which may have been treatment related was a statistically significant increase in the absolute mean cecum weight among females of the high-dose group compared to untreated controls. This increase in cecum weight was not seen in males, however, increases in cecum weight for male Wistar rats fed diets containing 20 or 40% pullulan has been reported (Oku et al., 1979). This effect, however, is regarded as physiological and is commonly observed with poorly digested starches/dextrins (Reussner et al., 1963), sugar alcohols and other fermentable carbohydrates (Clevenger et al., 1988; Mackenzie et al., 1986; Oliver et al., 1991).

The macroscopic changes found were consistent with those

commonly observed in these rats housed under the conditions of this study. Histological findings were in good agreement with macroscopic observations. None of the findings appeared to be related to treatment with pullulan.

Pullulan did not increase the number of mutants per plate of the Salmonella typhimurium tester strains, TA 100, TA 1535, TA 98 and TA 1537, either in the presence or absence of microsomal enzymes from Aroclor-induced rat liver. It is concluded that pullulan is not mutagenic with or without metabolic activation in the standard bacterial test system suggesting it lacks mutagenic/carcinogenic potential.

In conclusion, the only treatment-related effect found in these studies was a statistically significant increase in mean cecum weight for females fed 10% pullulan. Cecal enlargement is a generic response to poorly absorbed sugars and other carbohydrates in rats (Oliver et al., 1991), as for example, sorbitol or lactose (Mackenzie and Hauck, 1986). The enlargement is considered an adaptative rather than a pathologic change (El-Horith et al., 1976) As no other effects were noted which could be considered treatment related or adverse, it is concluded that pullulan lacks toxicologic activity. A NOAEL of 10% in the diet, the highest concentration tested, equal to 4450 mg/kg bw/day, will support an acceptable daily intake of at least 45 mg/kg bw/day as an ingredient of food.

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TABLE 1

Theoretical and Actual Pullulan Intake

Group (Dose Level) % diet	No. Rats M	No. Rats F	mg/kg bw/day Theoretical M	Theoretical F	Actual M	Actual F
0	15	15	0	0	0	0
1	15	15	500	500	480	520
5	15	15	2500	2500	2320	2630
10	15	15	5000	5000	4450	5080

TABLE 2

Final Body Weight of Sprague Dawley Rats

Fed Pullulan for 62 Weeks

Dose Level % diet	Body Weight (g) (S.D.) Males	Females
0	665 (36)	361 (58)
1	591 (81)*	414 (79)
5	640 (60)	335 (46)
10	606 (47)*	398 (58)

*P< 0.05

TABLE 3

Organ Weights (g) (S.D.) and Organ Body Weight Ratios in Male Rats Fed for 62 Weeks

	Dosage Level (% in diet)			
	0	1	5	10
Brain	1.80	2.05*	2.03	1.98
(S.D.)	(0.26)	(0.12)	(0.11)	(0.10)
Brain/b.w. x 100	0.27	0.34	0.32	0.33
Heart	1.69	1.56	1.73	1.52*
(S.D.)	(0.10)	(0.19)	(0.31)	(0.13)
Heart/b.w. x 100	0.26	0.26	0.27	0.25
Liver	20.8	18.0*	18.4*	17.6**
(S.D.)	(1.2)	(3.2)	(1.9)	(1.0)
Liver/b.w. x 100	3.14	3.03	2.88	2.90
L. Lung	1.13	1.12	1.00	1.12
(S.D.)	(0.20)	(0.46)	(0.22)	(0.55)
L.Lung/bw x 100	0.17	0.19	0.16	0.18
R.Lung	2.05	2.27	2.10	2.19
(S.D.)	(0.26)	(0.51)	(0.65)	(0.88)
R.Lung/b.w. x 100	0.31	0.38	0.33	0.36
Spleen	1.03	0.85	1.34	0.95
(S.D.)	(0.22)	(0.16)	(0.79)	(0.18)
Spleen/b.w. x 100	0.16	0.14	0.21	0.16
Stomach	2.51	2.29	2.37	2.24
(S.D.)	(0.26)	(0.23)	(0.23)	(0.18)
Stomach/b.w. x 100	0.38	0.38	0.37	0.37

Continued from Table 3

Testes	3.53	3.59	2.92	3.52
(S.D.)	(0.08)	(0.22)	(1.05)	(0.36)
Testes/b.w. x 100	0.53	0.60	0.46	0.58
L. Kidney	2.22	1.87*	2.00	1.83*
(S.D.)	(0.29)	(0.29)	(0.21)	(0.29)
L. Kidney/b.w. x 100	0.34	0.31	0.31	0.30
R. Kidney	2.25	1.90*	1.97*	1.80**
(S.D.)	(0.27)	(0.28)	(0.16)	(0.22)
R. Kidney/b.w. x 100	0.34	0.32	0.31	0.30
Adrenals	0.06	0.06	0.07	0.05
(S.D.)	(0.01)	(0.01)	(0.01)	(0.01)
Adrenals/b.w. x 100	0.01	0.01	0.01	0.01
Subman. Gland	0.81	0.77	0.74	0.64**
(S.D.)	(0.09)	(0.10)	(0.09)	(0.04)
Subman./b.w. x 100	0.12	0.13	0.12	0.11

*P< 0.05, **P< 0.01

TABLE 4

Organ Weights (g) (S.D.) and

Organ/Body Weight Ratios in

Female Rats Fed for 62 Weeks

	Dosage Level (% in diet)			
	0	1	5	10
Brain	1.75	1.65*	1.54**	1.62
(S.D.)	(0.13)	(0.04)	(0.13)	(0.13)
Brain/b.w. x 100	0.48	0.40	0.47	0.39
Heart	1.03	1.15*	1.06	1.20*
(S.D.)	(0.11)	(0.13)	(0.12)	(0.14)
Heart/b.w. x 100	0.28	0.28	0.32	0.29
Liver	11.9	13.2	10.7	14.3*
(S.D.)	(2.2)	(3.1)	(1.7)	(2.2)
Liver/b.w. x 100	3.25	3.22	3.23	3.43
L.Lung	0.70	0.80	0.74	0.72
(S.D.)	(0.18)	(0.12)	(0.19)	(0.08)
L.Lung x 100	0.19	0.20	0.23	0.17
R.Lung	1.43	1.56	1.42	1.47
(S.D.)	(0.50)	(0.41)	(0.37)	(0.12)
R.Lung/b.w. x 100	0.39	0.38	0.43	0.35
Stomach	1.78	1.59	1.70	1.91
(S.D.)	(0.20)	(0.54)	(0.31)	(0.24)
Stomach/b.w. x 100	0.49	0.39	0.51	0.46
Cecum	1.41	1.50	1.38	2.05**
(S.D.)	(0.27)	(0.36)	(0.30)	(0.45)
Cecum/b.w. x 100	0.39	0.37	0.42	0.50

Continued from Table 4

L. Kidney	1.06	1.29	1.13	1.27
(S.D.)	(0.32)	(0.29)	(0.17)	(0.13)
L.Kidney/b.w. x 100	0.29	0.31	0.34	0.30
R.Kidney	1.13	1.28	1.07	1.24
(S.D.)	(0.13)	(0.29)	(0.17)	(0.15)
R.Kidney/b.w. x 100	0.31	0.31	0.32	0.30
Adrenals	0.07	0.08	0.08	0.11
(S.D.)	(0.01)	(0.01)	(0.02)	(0.05)
Adrenals/b.w. x 100	0.02	0.02	0.02	0.03
Spleen	0.55	0.61	0.53	0.75**
(S.D.)	(0.10)	(0.16)	(0.19)	(0.14)
Spleen/b.w. x 100	0.15	0.15	0.16	0.18
Uterus	0.85	0.98	0.86	0.93
(S.D.)	(0.18)	(0.37)	(0.20)	(0.28)
Uterus/b.w.	0.23	0.24	0.26	0.22
Ovary	0.10	0.10	0.10	0.11
(S.D.)	(0.03)	(0.02)	(0.01)	(0.01)
Ovary/ b.w. x 100	0.03	0.03	0.03	0.03
Subman. Gland	0.52	0.56	0.54	0.78
(S.D.)	(0.08)	(0.05)	(0.07)	(0.44)
Subman. Gland/ b.w. x 100	0.14	0.14	0.16	0.18

*P< 0.05, **P< 0.01

TABLE 5

Results of Mutagenicity Test of Pullulan

Test Item	Dose μg/plate	Mutant Colonies/Plate TA100	TA1535	TA98	TA1537
Pullulan without S-9 mix	0	97	19	21	17
	10	86	12	30	5
	100	97	17	30	8
	1000	96	23	28	7
	10000	110	21	38	8
Pullulan with S-9 mix	0	110	7	39	11
	10	100	9	36	14
	100	100	18	29	12
	1000	100	12	32	14
	10000	94	5	27	13
Positive Control Test	SPOT TEST				
	with S-9 mix				
	Dimethylnitrosamine, 50 mg		+		
	without S-9 mix:				
	AF2, 0.05 mcg	+		+	
	9-Aminoacridine, 100 mcg				+

Figure-1. **Structure of Pullulan**

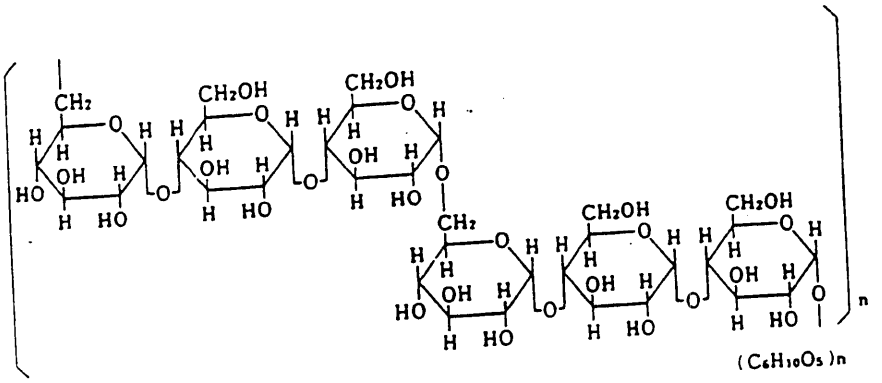

Figure 2

% Survival Curve - Males

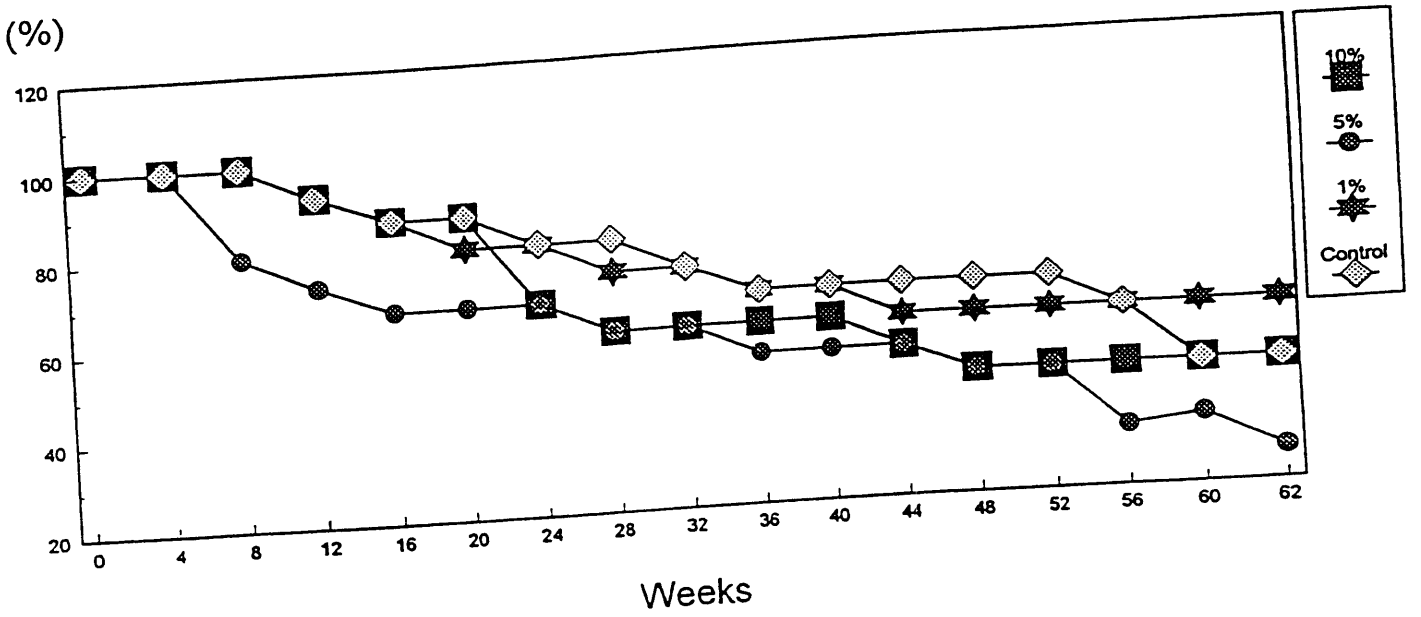

Figure 3

% Survival Curve - Females