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 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 $(1 \rightarrow 4)$ linked $\alpha$-D glucose molecules followed by a $(1 \rightarrow 6)$ linked $\alpha$-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 $1 \rightarrow 4$ glycosidic linkages (Catley et al., 1986; Tsujisaka and Mitsuhashi, 1993). Occasional branch points (Wallenfels et al., 1965) with $1 \rightarrow 3$ 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.

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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 cellulosics (JECFA, 1992; EC, 2003). According to the petitioner the substance is ≥90% pure calculated as the difference between total dry weight and the sum of the known impurities (e.g. ash, mono, di and oligosaccharides). 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 ≥ 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

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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

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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**

*Aureobasidum pullulans* (formerly *Pullularia pullulans*) is a non-pathogenic and non-toxigenic 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.*, 1994). 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 \( \alpha_1 \rightarrow 4 \) bond from the non-reducing end of the molecule and terminates at the \( \alpha_1 \rightarrow 6 \) 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). 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).

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 third. 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 (Hachiya et al. 1985, Kuroda et al. 1989). 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).

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).

**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

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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 $129 \pm 30$ g/day to $188 \pm 35$ g/day (146%) with pullulan and from $127 \pm 28$ g/day to $144 \pm 30$ 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 (Oku\textsuperscript{13} \textit{et al.}, 1983).

**Allergenicity and Immunogenicity**
Allergic alveolitis and hypersensitivity pneumonia has been linked to inhalation of \textit{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 \textit{Aureobasidium pullulans}, the source organism for pullulan production, leading to respiratory symptoms including hypersensitivity pneumonitis (Woodard \textit{et al.}, 1988; Apostolakos \textit{et al.}, 2001) and other lesions (Ajello, 1978). The fungus is also reported to be a frequent source of respiratory allergy (Kurup \textit{et al.}, 2000) and IgE antibodies to \textit{Aureobasidium pullulans} have been identified in sera from individuals with suspected mould allergy (Karlsson-Borga \textit{et al.}, 1989). Experimentally it has been demonstrated to cause extrinsic allergic alveolitis in the rabbit (Bulman, 1974).

\textit{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 \textit{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 \textit{Aureobasidium pullulans}) were considered “unlikely” to be of pathogenic significance (the remaining 7 (none \textit{Aurobasidium pullulans}) of “possible pathogenic significance”

Opportunistic infection with \textit{Aureobasidium pullulans} has been reported in immunocompromised patients by Salkin \textit{et al.} (1986), Kaczmarski \textit{et al.} (1986) and Giradi \textit{et al.} (1993).

\textsuperscript{13} Paper in Japanese, abstract and figure legends in English.
Over a 25 year period there have been no occurrences of allergic reaction to \textit{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.

\textbf{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 \textit{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. \textit{In vitro} studies suggest that PI-20 is broken down into smaller polymers (of around 70,000 daltons) by salivary and pancreatic amylases (Okada \textit{et al.} 1990). \textit{In vitro} and \textit{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 \textit{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

**REFERENCES**


Anonymous (1974 b). Report of acute toxicity test on pullulan with mice. Report of Department of Public Hygiene, School of Medicine, Juntendo University, Tokyo, for the petitioner, 1974, unpublished


**Scientific Panel Members**

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